STUDIES ON THE POTENTIAL USE OF CHLORINE DIOXIDE FOR TREATMENT OF SEAFOOD PRODUCTS: MICROBIOLOGY, NUTRITIONAL ANALYSIS, AND MUTAGENICITY

BY

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I dedicate this dissertation to my parents and to my wife
as a small token of my love and gratitude.

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Chlorine dioxide (ClO_2) has been considered a potential substitute for aqueous chlorine in treating seafood products. Its use for such treatment was examined on fillets of Atlantic salmon (*Salmo salar*), red grouper (*Epinephelus morio*), mahi-mahi (*Coryphaena hippurus*), headless brown shrimp (*Penaeus aztecus*), white penaeid shrimp (*Penaeus setiferus*), calico scallops (*Aequipecten gibbus*), sea scallops (*Placopecten magellanicus*) and gutted whole Atlantic salmon and red grouper to determine its impact on reducing bacterial loads and improving the quality of treated products.

Bacterial loads and the sensory quality of selected seafoods stored on ice were evaluated from 0-7 days. Compared to nontreated controls and the brine treated group, treatment of red grouper and salmon fillets, and whole salmon with ClO_2 solutions on day

0 caused dose-related but nonsignificant decreases in numbers of natural bacterial flora. Scallops, shrimp, and whole red grouper treated with ClO_2, however, showed significant decreases in bacterial numbers on day 0. Most samples treated and stored for 3 and 7 days also had decreased bacterial numbers with the higher ClO_2 treatment being significantly ($P<0.05$) different from all controls. Although the pH of ClO_2 solutions increased following the dipping of all samples, the solutions after treatment contained very low or undetectable bacterial loads. Discoloration of treated seafood resulted at 100 or 200 ppm concentrations, with the skins of red grouper and salmon becoming lighter in color. The occurrence of a chocolate color in the gills was a major drawback for using high ClO_2 concentrations for whole fish.

Thiobarbituric acid (TBA) values and fatty acid compositions were determined after ClO_2 treatments. Salmon and red grouper showed an increase in TBA; the 100 and 200 ppm groups had significantly greater TBA values than did the controls and 20 ppm group. Red grouper and salmon at 100 and 200 ppm ClO_2 did not differ in percent monounsaturated and polyunsaturated fatty acids compared to controls, although differences occurred with some individual fatty acids. Thus, ClO_2 treatment did not greatly affect fatty acid composition. Protein content of salmon and red grouper was also unaffected by ClO_2 treatments. However, ClO_2 caused significant reductions in thiamine for salmon and red grouper, and riboflavin for red grouper only. Levels of calcium, iron and phosphorus in red grouper fillets and phosphorus and potassium in salmon fillets were not affected following treatment with ClO_2 solutions.

Low levels of chlorate residues were detected in some of the treated seafood samples, but no chlorite residues were found in any of the treated seafoods. Extracts from ClO_2 treated solutions and treated fish fillets were found to be non-mutagenic to *Salmonella typhimurium* strains TA 98 and TA 100 with or without the S-9 mix. Since ClO_2 is a more potent bactericidal agent than chlorine and non-mutagenic, it can be used as a substitute for chlorine in the treatment of seafood to enhance freshness, extend shelf-life, and improve the safety of the products.

CHAPTER I
INTRODUCTION

Seafood safety and quality have been the major targets of consumer groups and professional authorities in the United States. Nationally, seafood, shellfish and aquaculture products have been under siege by continuing reports of microbial and toxin contamination problems. Professional associations, regulatory agencies and trade associations have been actively searching for effective methods to maintain the safety and quality of seafood. Due to the perishable character of seafood, the industry has long applied aqueous chlorine solutions for washing fish, shrimp, crab, clam, and oyster to meet microbiological standards, prevent spoilage, and increase shelf-life because of its disinfecting capability. Chlorine has been used as a direct or indirect food additive because of its GRAS (Generally Recognized as Safe) status designated by the Food and Drug Administration (FDA).

Safety concerns were raised, however, when chlorine was reported to react with organic matter to form low levels of hazardous by-products. Many of these are oxidation or halogenation reaction products of naturally occurring organic substances present in water or food. These chloro-organics and oxidized products may pose health hazards to humans following long term exposure. Concern about the safety of chlorine as a disinfectant for potable water treatment arose when undesirable trihalomethanes (THMs) were discovered as a result of water chlorination practices (Bellar et al., 1974). These concerns were

exacerbated when the National Cancer Institute confirmed that chloroform, a major THM, was carcinogenic to laboratory animals (National Cancer Institute, 1976). The formation of undesirable toxic reaction products during chlorination to produce potable water and the need to reduce total THM levels in drinking water below 0.10 mg/L have led to a search for alternative disinfectants (U.S. Environmental Protection Agency, 1975).

Chlorine dioxide (ClO_2) is a potent bactericidal agent with an effect equivalent to seven times its concentration of chlorine in poultry processing water (Lillard, 1979). The bactericidal activity of ClO_2 decreases with lower temperatures (Ridenour and Ambruster, 1949), but is not affected by high pH or the presence of ammonia or nitrogenous compounds (White, 1972). Though ClO_2 maintains its bactericidal activity far longer than chlorine, it is less reactive than chlorine with organic compounds such as unsaturated fatty acids and their methyl esters (Wei et al., 1987), and tryptophan and its derivatives (Sen et al., 1989). The consequence is a reduction in the formation of potentially toxic chlororganic compounds. Chlorine dioxide is thus used preferentially when high organic loads are encountered (White, 1972); it has proven to be a more effective bactericide than chlorine in sewage effluent at pH 8.5 (Benarde et al., 1967).

In this country, ClO_2 has not been approved for use in the processing of seafood or red meat, nor has it been approved for the treatment of food processing water for reuse. Regulatory approval is lacking due to the limited availability of research data related to the nature and toxicity of the reaction products, and the effect of such treatments on the nutritional value of treated foods. Although considerable efforts have been made to study the antimicrobial activity of ClO_2 with broiler carcasses, there is little research information

available regarding its effect on reducing seafood microflora and pathogens and on the potential health hazards related to such application. Thorough research is therefore needed to assess the efficiency of ClO_2 in controlling microbial flora on seafood and the toxicity of reaction products following treatment in a processing situation.

In this study, efforts were made to study the bactericidal activity of ClO_2 and the impact of such treatment on seafood organic components as well as the potential toxicity of the reaction products generated. The Ames *Salmonella*/microsome assay was applied to evaluate the potential mutagenic activity of reaction products present in the solutions and on the surfaces of treated seafoods. Thus, the objective of this study was to investigate the overall usefulness and safety of ClO_2 as a treatment for seafood products.

Since ClO_2 may react with seafood organic components to produce reaction products and affect the chemical composition and nutritional value of seafood samples, the specific aims of this study were as follows:

Aim 1: Determination of the antimicrobial efficiency of ClO_2 in killing/retarding natural seafood microflora and the effectiveness in extending the shelf-life of seafood products (red grouper and salmon [whole fish, gutted; and fillets], scallops, and shrimp) following treatment with ClO_2 solutions at 20, 40, 100, and 200 ppm total available chlorine dioxide (TACD).

Aim 2: Determination of the effect of treatment with aqueous ClO_2 solutions on total lipid oxidation and fatty acid composition in red grouper and salmon fillets.

Aim 3: Determination of the effect of ClO_2 treatment on the nutritional composition of salmon and red grouper fillets and analysis of residual available ClO_2.

Aim 4: Determination of the effect of ClO_2 treatment at more practical levels (10, 20 and 30 ppm) on the quality, bacterial loads, and inorganic anions of seafoods (sea scallop, shrimp, and mahi-mahi).

Aim 5: Determination of potential mutagenic reaction products derived from treated seafood or solutions following treatment of salmon and red grouper with aqueous ClO_2 solutions.

The contents presented in chapters III-VII each compose a complete study. The intent is to publish each as an individual paper with specific objectives, methods of approaches, and defined results and discussion. The last chapter (VIII) summarizes the findings of this doctoral research. Recommendations for future studies are also included in this chapter.

CHAPTER II
REVIEW OF THE LITERATURE

Historical Perspectives

Chlorine is an element of the halogen family, but is never found uncombined in nature. It is estimated to account for 0.15% of the earth's crust in the form of soluble chlorides, such as common salt (NaCl), carnallite ($KMgCl_3 \cdot 6H_2O$), and sylvite (KCl). In nature, therefore, it exists only as the negative chloride ion with a valence of -1 (White, 1972). A brief history of chlorine or chlorine dioxide was described by the articles of White (1972), Aieta and Berg (1986), and Dychdala (1991). Chlorine is believed to have been known to medieval chemists long before it was discovered in its gaseous state in 1774 by a Swedish chemist named Karl W. Scheele. Scheele also observed that the gas was soluble in water, that it had a permanent bleaching effect on paper, vegetables, and flowers. Humphry Davy, in 1810, declared the gas to be an element and named it chlorine, derived from the Greek word "chlorous" which has been translated to mean green, greenish yellow or yellow green, based on its color. In 1811, Davy discovered chlorine dioxide gas when he reacted potassium chlorate ($KClO_3$) with sulfuric acid (H_2SO_4). The gas had an intense color similar to that of chlorine and subsequently named euchlorine. The first reference in the literature to chlorite was that of Millon, who obtained a green-yellow gas by acidifying potassium chlorate with hydrochloric acid in 1843. Millon's gas, as it came be known, was

not recognized to contain chlorine dioxide (ClO_2) until 1881 when Garzarolli-Thurnlackh identified the gas as a mixture of ClO_2 and chlorine. In 1940, Taylor et al. discussed the release of chlorine dioxide gas from sodium chlorite upon acidification or reaction with chlorine (Aieta and Berg, 1986).

Even though the bleaching action of chlorine had been used for a long time, the disinfecting and deodorizing properties were not known until early in the nineteenth century. Chloride of lime was first introduced to North America by Johnson in 1908 for water purification, and within a few years over 800 millions gallons of water had been purified by chlorination (Dychdala, 1991). In 1881, a German bacteriologist, Koch, demonstrated the bactericidal action of chlorine as hypochlorite on pure bacterial cultures under controlled laboratory conditions. In 1886, the American Public Health Association issued a favorable report on the use of hypochlorite solution as a disinfectant (Hadfield, 1957). In 1894, Traube established the first guidelines for hypochlorite in water purification and disinfection (Dychdala, 1991; Hadfield, 1957). To date, chlorine has gained worldwide recognition as the disinfectant of choice for treatment of potable water. It was not until 1944 that ClO_2 was first used for water treatment at a facility in Niagara Falls, New York. The use of ClO_2 in this instance resulted because of the availability of a large inventory of sodium chlorite for military use during World War II.

Chlorine dioxide is widely used in Europe as an alternative to chlorine for drinking water disinfection. It is currently used in approximately 500 drinking water treatment plants in the U.S., including such cities as Philadelphia, Shreveport, El Paso, and Galveston (Latshaw, 1994). Among the states in which ClO_2 is most extensively used are Georgia,

Ohio, Pennsylvania, and Michigan. The largest user of ClO_2 today is the paper pulp industry, where ClO_2 is used extensively as a bleaching agent (Pryke, 1989).

The dairy industry was the first food processing industry to utilize the disinfecting and deodorizing properties of chlorine (Mercer and Somers, 1957). In this respect, chlorine was used as a sanitizer for milk bottles and equipment. Use of chlorine spread to other food industries. It is used in all branches of the food processing industry including canning, flour, poultry, fish, meat, fruits and vegetables. However, safety concerns were raised when chlorine was reported to react with organic matter to produce low concentrations of potentially hazardous byproducts, oxidation or halogenation reaction products of naturally occurring organic substances present in water or food. Chlorine dioxide, therefore, has been considered as a potential substitute of aqueous chlorine.

Chemical Properties of Chlorine Dioxide

Chlorine dioxide is a yellow-green gas similar in appearance and odor to chlorine. Unlike chlorine, ClO_2 gas cannot be compressed and bottled because of its explosiveness. Concentrations in excess of 10% ClO_2 in air are apt to undergo mild explosions under the influence of heat, light or shock. Consequently, ClO_2 must be generated on-site. As a polar gas, ClO_2 is quite soluble in water depending on temperature and pressure. In aqueous solution, ClO_2 is quite safe. Relevant chlorine species for this discussion and solubility of ClO_2 are shown in Table 1 and Figure 1, respectively.

Table 1. Relevant chlorine species

Chemical formula	Name
Cl_2	Molecular chlorine (gas)
HOCl	Hypochlorous acid
OCl^-	Hypochlorite ion
Cl^-	Chloride ion
Cl^+	Chloronium ion
ClO_2^-	Chlorite ion
ClO_3^-	Chlorate ion
$NaClO_2$	Sodium chlorite
$NaClO_3$	Sodium chlorate
ClO_2	Chlorine dioxide (gas)
ClO	Monochlorine monoxide
$HClO_2$	Chlorous acid
$HClO_3$	Chloric acid

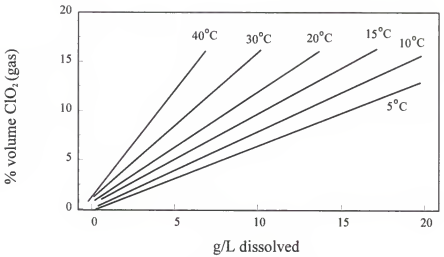


Figure 1. Solubility of chlorine dioxide in water (Adapted from Masschelein, 1979)

The solubility of ClO_2 in water is greater than that of chlorine. Chlorine dioxide in solution theoretically is a mixed anhydride of chlorous acid ($HClO_2$) and chloric acid ($HClO_3$):

$$2\ ClO_2 + H_2O \rightarrow HClO_2 + HClO_3 \quad (2H^+ + ClO_2^- + ClO_3^-)$$

White (1972) reported that ClO_2 does not disproportionate in water. When the pH is practically neutral, ClO_2 dissolves in water with almost no hydrolysis. It appears that this disproportionateness is rate dependent on pH (Tabue and Dodgen, 1949). Gordon et al. (1972) stated that at strongly alkaline pH, ClO_2 disproportionates rapidly to give equal amounts of ClO_2^- and ClO_3^-: $2\ ClO_2 + 2\ OH^- \rightarrow ClO_2^- + ClO_3^- + H_2O$. However, in an extensive review of the chemistry of ClO_2, they concluded that this disproportionateness had not been sufficiently investigated. Chlorine dioxide is readily decomposed on exposure to UV light and, therefore, it is always stored in the dark. It is also extremely volatile. Although the oxidizing potential of ClO_2 is less than that of HOCl, it has a larger oxidation

capacity because it can accept 2.5 times more electrons (White, 1972). Chlorine dioxide is 52.6% chlorine by weight. In the process of reduction to Cl^-, ClO_2 undergoes five reduction steps:

$$ClO_2 + 5e^- \rightarrow Cl^- + 2O^{2-}$$

In its process of reduction to Cl^-, Cl_2 undergoes one reduction step:

$$\frac{1}{2} Cl_2 + e^- \rightarrow Cl^-$$

In terms of oxidation capacity, 1.0 mg/L of ClO_2 equals 2.63 (0.526 x 5) times the oxidizing power of chlorine. However, the oxidizing potential of ClO_2 is less than HOCl as indicated by its redox potential (Masschelein, 1979):

$$ClO_2 + e^- \rightarrow ClO_2^- \qquad + 0.95 \text{ V}$$

$$ClO_2^- + 2H_2O + 4e^- \rightarrow Cl^- + 4OH^- \qquad + 0.78 \text{ V}$$

In water treatment involving ClO_2, the chloro-species might include ClO_2, free Cl_2, and combined Cl_2 (ClO_2^- and its acid $HClO_2$; ClO_3^- and its acid $HClO_3$ and Cl^-).

Many organic compounds can be oxidized and chlorinated by ClO_2, but at a much slower rate than that associated with HOCl reactions. That is because ClO_2 is less reactive than chlorine with organic compounds such as unsubstituted aromatics and primary amines (Raugh, 1979), unsaturated fatty acids and their methyl esters (Wei et al., 1987), and tryptophan and its derivatives (Sen et al., 1989). Another advantage of using ClO_2 is that no trihalomethanes (THM) have been detected as reaction products of ClO_2 with organic materials (Moore et al., 1980) ; ClO_2 does not react with ammonia commonly found in water to form chloramines (White, 1972).

The mechanisms of chlorination and oxidation of organic compounds by ClO_2 are not well known. Chlorination in aqueous solutions may occur indirectly through a progressive reduction of ClO_2, which passes through the HOCl stage:

$$ClO_2 + e^- \rightarrow ClO_2^-$$

$$ClO_2^- + 2e^- + 3H^+ \rightarrow HOCl + H_2O$$

However, the mechanisms of chlorination by ClO_2 and the above transformations are far from being resolved (Masschelein, 1979).

In contrast, when chlorine gas is dissolved in water it rapidly hydrolyzes (actually reacts with the H_2O molecule) to form hypochlorous acid (HOCl):

$$Cl_2 \text{ (gas)} + H_2O \rightarrow HOCl + H^+ + Cl^-$$

The further dissociation of hypochlorous acid follows to produce hypochlorite ion (OCl^-) :

$$HOCl \rightleftharpoons H^+ + OCl^-$$

The resulting pH of the water into which the chlorine gas is dissolved determines the speciation of chlorine as shown in Figure 2 for a 0.05 M chlorine solution (this is the highest chlorine concentration encountered in typical installations).

The relative concentrations of molecular chlorine (Cl_2) and HOCl are governed principally by the pH of the system. The ratio of OCl^- to HOCl increases as the pH increases. At pH values greater than 7.5, OCl^- becomes the dominant species in an aqueous chlorine solution at 25 ºC (White, 1972). All three species, Cl_2, HOCl and OCl^-, will readily oxidize organic compounds. However, HOCl is the most reactive as reflected by its higher redox potential (Masschelein, 1979):

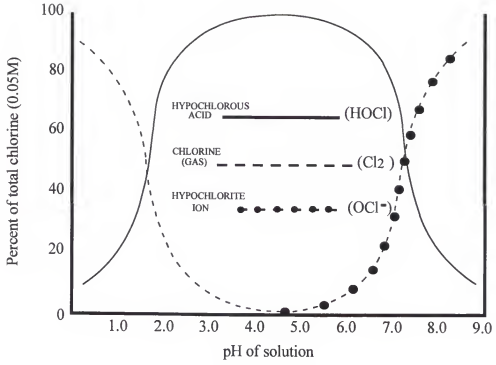


Figure 2. Calculated percentage of chlorine species in aqueous solution as influenced by pH (Adapted from Foegeding, 1983)

$HOCl + H^+ + 2e^- \rightarrow Cl^- + H_2O$ + 1.49 V

$Cl_2 + 2e^- \rightarrow 2Cl^-$ + 1.36 V

$OCl^- + H_2O + 2e^- \rightarrow Cl^- + 2OH^-$ + 0.90 V

The reactivity of HOCl is based on the electrophilic nature of the molecule at either the oxygen or chlorine atom (Morris, 1978). Reactions occurring at the oxygen atom produce a chloride ion (Cl^-) by displacement. In reactions with organic compounds, the chlorine atom becomes the electrophilic agent, taking on the characteristics of Cl^+, and may combine with an electron pair in the substrate. The possible chemical reactions with organic

constituents in aqueous solutions may be grouped into several general types according to Jolley (1978) (Table 2). However, ClO_2 generally reacts as an electron acceptor, and H atoms present in activated organic C-H or N-H structures are thereby not substituted by Cl.

Table 2. Principal organic reactions of hypochlorous acid with organic compounds

Organic reactions	Example
Oxidation	$RCHO + HOCl \rightarrow RCOOH + H^+ + Cl^-$
Addition	$RHC{=}CHR' + HOCl \rightarrow RCH(OH)CH(Cl)R'$
Substitution	
N-Cl Bond	$RNH_2 + HOCl \rightarrow RNHCl + H_2O$
C-Cl Bond	$RCOCH_3 + 3HOCl \rightarrow RCOOH + CHCl_3 + 2H_2O$

(Adapted from Jolley et al., 1978)

Methods for Generating Chlorine Dioxide and for Determining Chlorine Compounds

Chlorine dioxide can be prepared either by reduction of chlorates or by oxidation of chlorites. The large scale commercial operation of bleaching pulp with ClO_2 uses reduction of chlorate. There are several methods by which ClO_2 may be efficiently produced. Generally, all industrial scale reactions leading to ClO_2 may be applied in the laboratory, including:

1) Reduction of chlorates by sulfurous anhydride

$$2\ NaClO_3 + H_2SO_4 + SO_2 \rightarrow 2\ ClO_2 + 2\ NaHSO_4$$

The addition of reducing agents in the industrial synthesis of ClO_2 with chlorates increases yields and enables the direct manufacture of ClO_2 of even higher purity than that obtained by a simple acidification process. In practice, SO_2 is the most frequently used

reducing agent. Reducing agents other than SO_2 used for the synthesis of ClO_2 from chlorates are methanol, nitrites, NO_2 and chromium or manganese salts.

2) The electrochemical reduction of chlorates

The electrochemical reduction of chlorates to ClO_2 corresponds to the following probable equation:

$$NaClO_3 + 2\ HCl \rightarrow ClO_2 + ½\ Cl_2 + H_2O + NaCl$$

3) Acidification of chlorite

For laboratory purposes the acidification of a solution of sodium chlorite is the most practical. At a pH of 3.5, the chlorite undergoes a slow transformation with disproportion to ClO_2, chlorate and chloride.

$$5\ NaClO_2 + 4\ HCl \rightleftharpoons 4\ ClO_2 + 5\ NaCl + 2\ H_2O$$

The acids used in practice are hydrochloric acid, sulfuric acid, citric acid, acetic acid, and phosphoric acid.

4) Oxidation of chlorite by chlorine

$$2\ NaClO_2 + Cl_2 \rightarrow 2\ NaCl + 2\ ClO_2$$

5) Oxidation of chlorite by persulfate

$$2\ NaClO_2 + Na_2S_2O_8 \rightarrow 2\ ClO_2 + 2\ Na_2SO_4$$

6) Action of acetic anhydride on chlorite

$$NaClO_2 + (CH_3CO)_2O + H_2O \rightarrow ClO_2 + CH_3COOH + CH_3COONa + H^+$$

Besides the well-established methods of preparation and synthesis of chlorine dioxide, there are several other alternatives that have not yet found real application.

The literature indicates that several analytical procedures for the determination of ClO_2 in drinking water are available: an iodometric method (Ingols and Ridenour, 1948; Granstrom and Lee, 1958; Post and Moore, 1959) or a colorimetric method using orthotolidine (APHA, 1989), tyrosine (Hodgen and Ingols, 1954), and *N,N'*-diethyl-*p*-phenylenediamine (DPD) (Palin, 1960, 1967). The United States Public Health Services (USEPA Analytical Reference Service, 1969, 1971) conducted a comparative study of the methods and concluded that the DPD titrimetric method showed the best overall results. Also, Adams et al. (1966) and Ward (1976) reported that DPD was the most reliable and was the procedure of choice.

Reactions of Chlorine and Chlorine Dioxide with Organic Compounds Found in Foods

A wide variety of organic materials including those components of food systems such as carbohydrates, proteins, fatty acids, and vitamins, are subjected to oxidation and chlorination reactions in aqueous chlorine and ClO_2 solutions. Not mentioned are many other organic chemicals present in food that may also react with chlorine or ClO_2. These include nucleic acids, vitamins and vitamin precursors, complex lipids, steroids, other chemicals and essential trace elements, as well as food additives such as antimicrobial agents and antioxidants. The amino acids are of particular interest as targets for chlorine reactions because of their relative abundance in water (Scully et al., 1988).

In spite of its chemical similarity to chlorine, ClO_2 reacts with organic materials by a different mechanism. Whereas chlorine can react via several possible mechanisms, including addition, substitution, and oxidation, ClO_2 primarily reacts with organic

compounds as an electron-transfer oxidant with the formation of oxygenated products such as diols, aldehydes, ketones, and carboxylic acids (Rav-Acha et al., 1986; Choshen et al., 1986).

Reaction with amino acids, peptides and proteins

Chlorine dioxide has been proposed as an alternative to chlorine for disinfection purposes. It is, therefore, important to understand the kind of reactions and by-products likely to be formed with its use, especially proteinaceous material. In reactions involving ClO_2 with amino acids, ClO_2 is reduced to chlorite (ClO_2^-) or the Cl^- and the amino acid is oxidized (White, 1972). Amino acids at low pH are expected to be more inert towards oxidation because of the presence of an electron-deficient center on the amino-nitrogen atom.

In combinations of equimolar concentrations of twenty-two amino acids and four nucleic acids with ClO_2 at neutral pH, only seven (cysteine, cystine, proline, histidine, hydroxyproline, tyrosine and tryptophan) were reactive (Noss et al., 1983). Chlorine dioxide is relatively inert towards individual amino acids when compared with aqueous chlorine. Additional evidence in support of these studies found that only six of twenty-one amino acids were reactive with ClO_2 (Tan et al., 1987). Three amino acids (cysteine, tryptophan, and tyrosine) reacted with ClO_2 at pH 3. At pH 6, three additional amino acids (histidine, hydroxyproline and proline) were reactive. At pH 9, methionine was also reactive (Tan et al., 1987).

From the above studies, it is clear that the reactivity of ClO_2 with amino acids and proteins depends on pH, temperature and contact time, with higher temperatures, contact times, and pH favoring the reaction. For example, glycine and phenylalanine were inert

toward ClO_2 at room temperature when equimolar concentrations of reagent and substrate were used (Noss et al., 1983). The sulfur-containing amino acids and those with phenyl or heterocyclic rings were reported to be the most reactive with ClO_2. Of the amino acids containing sulfur, cystine has been reported to produce cysteic acid with a cystine disulfoxide intermediate (Masschelein, 1979). The reaction is said to involve the breakage of the sulfur-sulfur bond of cystine followed by further oxidation. Under similar conditions, methionine is oxidized to sulfoxide and then to sulfone (Masschelein, 1979).

Chlorine dioxide oxidation of tryptophan produced indoxyl, isatine, indigo red as well as unidentified polymerization products. Structurally similar indole derivatives such as indole itself, 3-methyltryptophan, 3-indolelactic acid and 3-methylindole have also been shown to be readily oxidized by ClO_2, sometimes leading to color formation and precipitation (Lin and Carlson, 1984). Chlorine gas and ClO_2 have traditionally been used to treat flour to improve baking properties and dough quality. Wheat flour treated with high levels of ClO_2 did not produce chlorinated derivatives of amino acids or proteins (Meredith et al., 1956). However, the levels of cysteine and tryptophan were decreased by 25 and 8%, respectively. When flour was treated with ten times the commercial level of Cl_2, the levels of methionine, cysteine, tyrosine, and histidine decreased by 68.3%, 40.8%, 31.6%, and 16.8%, respectively. Other changes observed when wheat flour was exposed to high levels of ClO_2 included an increase in water solubility of the flour proteins. When solutions of wheat proteins were exposed to ClO_2, a pink-yellow color developed as a result of oxidation of tryptophan and tyrosine (Ewart, 1968). According to Tan et al. (1987) reactions involving ClO_2 with peptides (L-glycyl-L-tryptophan and L-trypto-phylglycine) and proteins (bovine

serum and casein) in phosphate buffer at pH 6 were too rapid for measuring the rates of disappearance of available chlorine by the iodometric method. However aspartame (L-aspartyl-L-phenylalanine methyl ester) did not react with ClO_2, possibly due to the presence of the methyl ester in this dipeptide. Proteins are large aggregates of peptides, and each peptide unit may be responsible for the loss of available chlorine.

Reaction with lipids

Lipids constitute a class of organic compounds in foods that readily react with chlorine. Aqueous chlorine completely removes double bonds in triolein, the major reactions being addition of chlorine and hydroxyl groups to the double bonds. Chlorine dioxide also reacts with triolein, modifying 25% to 50% of the double bonds present (Leopold and Mutton, 1959).

The reactivities of lipids with active species of chlorine are primarily due to the double bonds present in the free or esterified fatty acids. Double bonds can undergo oxidation and addition in the presence of electrophiles such as HOCl and ClO_2. Various lipids were reacted with $HO^{36}Cl$ and $^{36}ClO_2$ in an aqueous medium, and the extent of incorporation of ^{36}Cl into the lipids was determined (Ghanbari et al., 1982). Chlorine incorporation into lipids treated with HOCl was greater than lipids treated with ClO_2. Free fatty acids incorporated more Cl than their corresponding esters. The degree of Cl incorporation was directly correlated with number of double bonds in the lipid. Triglycerides behaved as esters of their component fatty acids. HPLC studies indicated at least 2 chlorinated products were formed from the reaction of $HO^{36}Cl$ with oleic acid (Ghanbari et al., 1982). Reaction of ClO_2 with methyl oleate, like aqueous chlorine, resulted in oxidation

and chlorination across the double bonds (Lindgren and Svahn, 1966). However, the levels of chlorine incorporation into methyl oleate were much less than that with aqueous chlorine. In addition, unsaturated ketones and chloroketones were the result of a different reaction mechanism than that of aqueous chlorine. These products resulted from the formation of allyl radicals rather than from direct addition to the double bonds. Highly volatile and soluble reaction products from ClO_2 and lipid reactions have not been tested for mutagenicity.

Reaction with carbohydrates

Reaction of Cl_2 (g), aqueous chlorine, or ClO_2 with carbohydrates generally results in oxidation products. In the reaction with Cl_2(g) or aqueous chlorine, glucose is oxidized at carbons C-2 and C-3 to form ketones. Further oxidation results in cleavage of the C-2 to C-3 bond and formation of carboxylic acids (Johnson et al., 1980). Aqueous ClO_2 will also react with glucose by oxidation of the hydroxy groups to form carbonyl derivatives, which are subsequently oxidized to carboxylic acids (Flis et al., 1966). Reaction products of ClO_2 and glucose have not been identified, however, β-D-glucose reacts more rapidly with ClO_2 than α-D-glucose.

The toxicological significance of chlorine-modified carbohydrates is not known. The potential formation of halocarbons from carbohydrates raises some concern about their production in treated flour and other food products. There is no evidence to indicate that other oxidation products are toxic after chronic exposure.

Disinfection by Chlorine Dioxide

Even though the bactericidal properties of ClO_2 were known at the beginning of this century, practical use of ClO_2 was not achieved until the middle of the century when ClO_2

was first applied to water treatment. Increased concerns over environmental and water pollution by chlorination reaction products also led to a search for alternative disinfectants (Masschelein, 1979). The bactericidal efficiency of ClO_2 was unaffected over a wide pH range (from pH 6-10), which makes it suitable for disinfection of high pH waters.

The effects of ClO_2 dose level, contact time, and pH on killing total coliforms, fecal coli, *Streptococcus*, total count, and *E.coli* phage were studied (White, 1992). Effluent disinfection with 2.7 mg/L ClO_2 showed a 98.9% kill of fecal coli after a 30 min contact time. This small dose did not to kill efficiently after a 5 min contact time. The killing efficiency was improved and contact times were shortened by increasing the ClO_2 dose levels. A dose of 10.8 mg/L ClO_2 was sufficient to reduce the fecal coli from 3.3×10^7 CFU/mL in effluents to 14 CFU/mL within 20 min.

Most studies regarding ClO_2 disinfection have focused on a comparison with chlorine and, as a result, very little information is available on its mode of action. Some of the comparative studies have shown that ClO_2 is a more efficient virucide and bactericide than chlorine.

Hypochlorous acid and ClO_2 were equally effective at 0.75 mg/L against *E. coli* at pH 6.5 in organic free buffer at 24 °C (Benarde et al., 1965). However, increasing the pH to 8.5 altered their relative disinfecting capabilities markedly. This was consistent with the knowledge that pH determines the degree of ionization of hypochlorous acid to hypochlorite ion, which is a markedly less effective bactericidal agent than the unionized acid. At pH 6.5, essentially all of the chlorine is present as hypochlorous acid and is potentially bactericidal. At pH 8.5, a major fraction of the chlorine is present as the hypochlorite ion and, in equal

concentrations, requires markedly longer contact periods to produce equal killing effectiveness. By contrast, ClO_2 does not ionize in water and its bactericidal efficiency remains substantially constant over the normal pH range of natural waters. Bactericidal rates were found to increase markedly with increased temperatures in the range of 5-32 °C (Benarde et al., 1967).

The theory of bacterial inactivation with chlorine and related compounds most widely accepted today is the reaction with or oxidation of the sulfhydryl groups (-SH) on protein. It is believed that the major proteins of interest are enzymes involved in important cell functions. Oxidizing agents may progressively oxidize sulfhydryl groups to disulfides and even to sulfoxides or sulfones (Gordon et al., 1972). Chlorine dioxide appears to oxidize the thiol group to sulfoxide or sulfone, a reaction which is biologically, at least, irreversible. After the oxidative change on the protein, the bacterial cell can not restore the functional sulfhydryl group. The relative complexity of ClO_2 action is, in fact, a manifestation of nonspecificity. In many instances, differences in susceptibility are observed to be due to the location of sulfhydryl groups rather than the intrinsic sensitivity of the protein. It could be expected that membrane-bound enzymes would appear to be more sensitive than those situated in the cytoplasm.

The proposed mechanisms for the action of ClO_2 on microbes are similar to those of chlorine, either through a reaction with macromolecules or through gross physiological changes (Roller et al., 1980). Many enzymes, including succinic dehydrogenase, have been shown to be located in bacterial cytoplasmic membrane. Damage to the membrane can, therefore, result in both inhibition of key enzymes and/or changes in permeability of the cell

with possible disastrous consequences. Since the high specificity of an enzyme is due to the unique surface contour of its tertiary structure and its distribution of charges on this contour, it is not difficult to imagine that any rearrangement of structure can easily upset its function. The results indicated that protein synthesis may be a contributing factor of cell death but some other essential function of the cell is apparently impaired first (Roller et al., 1980).

Chlorine dioxide is reactive towards several compounds including phenols, some amino acids and proteins. This reactivity has been used to explain its bactericidal action. On the mode of bacterial killing action, ClO_2 was sufficiently reactive with several amino acids to alter their structures and, thus, react with cell walls. Also, it was observed that protein synthesis was abruptly inhibited by the action of ClO_2 (Benarde et al., 1967). Chlorine dioxide readily and rapidly reacted with the amino acids cysteine, tyrosine, and tryptophan but not with viral RNA (Noss et al., 1983). Researchers postulated that the virucidal activity of ClO_2 was probably due to alteration of viral proteins. Chlorine dioxide reaction with tyrosine appeared to be the main mechanism of action for the F2 virus inactivation (Olivieri et al., 1985).

Another probable mechanism of action by ClO_2 may be its interaction with membrane lipids and fatty acids leading to permeability changes in membranes. Chlorine dioxide was highly reactive towards free fatty acids to form oxidized byproducts (Ghanbari et al., 1982). Free fatty acids were more reactive with ClO_2 than their methyl esters. It is possible that oxidation of membrane lipids and proteins by ClO_2 leads to microbial death. Even though some of the reactions have been reported to occur in model systems, the actual mechanism taking place in the intact microorganism stills need to be proven.

Health Hazards and Toxicity of Chlorine Dioxide

The first report concerning ClO_2 toxicity was made in 1955 by Haller and Northgraves. They demonstrated that rats exposed to 100 mg/L ClO_2 for a period of two years showed an increased mortality rate, whereas exposure of rats to 10 mg/L ClO_2 did not result in any adverse health effects. Subsequent studies by Fridlyand and Kagan (1971) revealed that rats treated with 10 mg/L ClO_2 for 6 months did not exhibit any adverse effect with respect to total body weight, relative organ weight, activity of oxidase enzyme (e.g., catalase), and vitamin C level in internal organs. At 100 mg/L ClO_2, only a decreased rate of weight gain was observed in the treatment group. Human studies conducted by Lubbers et al. (1982) showed no definitive finding of physiological impairment after acute and subchronic (21 days) ingestion of 5 mg/L ClO_2 by normal healthy adult males. An epidemiologic study was performed on humans exposed for three months to drinking water disinfected with ClO_2 in southeastern Ohio (Michael et al., 1981). There was no association between the use of ClO_2 treated water and clinical chemistry measurements.

The toxicology of ClO_2 as well as its inorganic byproducts, chlorate (ClO_3^-) and chlorite (ClO_2^-), has been studied extensively because of their observed effects on the hematopoietic system (Abdel-Rahman et al., 1984). All three compounds are usually evaluated together since both ClO_2 and chlorate can rapidly be converted to chlorite; therefore, some aspects of their toxicology are expected to be similar. *In vitro* studies demonstrated that chlorite produced methemoglobinemia in human and rat blood with virtually identical dose-response relationships (Heffernan et al., 1979). The toxic effect of chlorite on red blood cells was due to substantial oxidative damage to the cell membrane.

This membrane damage was accompanied by depletion of glutathione concentration and increased concentrations of hydrogen peroxide (Heffernan et al., 1979). Studies conducted in monkeys indicated that sodium chlorite induced dose-dependent oxidative stress on hematopoiesis, causing decreased hemoglobin and red cell count and increased methemoglobin content (Bercz et al., 1982).

The primary method of water disinfection in the United States employs chlorine. However, studies have shown that chlorine interacts with organics in the water to form trihalomethanes (THMs) (Rook, 1976). One of the THMs is chloroform, which has been found to be carcinogenic in rats (NCI, 1976). It has been postulated that chlorinated water supplies may increase the risk of cancer to humans (Marx, 1974). Such potential carcinogenic compounds may not be formed in water disinfected with ClO_2 (Miltner, 1977). The potential toxicity of ClO_2 and its metabolites (chlorate and chlorite ions) is of interest from a human health perspective. Chlorite is a strong oxidant that produces markedly exaggerated effects *in vitro* on red cells from glucose-6-phosphate dehydrogenase deficient humans (Moore et al., 1980). Abdel-Rahman et al. (1979) and Taylor and Pfohl (1984) demonstrated that ClO_2 decreased rat blood glutathion (GSH) concentration, and depressed DNA synthesis in testis and intestinal epithelium of rats. Glutathione is a tripeptide (γ-L-glutamyl-L-cysteinyl-glycine) that occurs in many cells in a variety of species. It is important to maintain adequate intracellular levels of GSH since this compound protects hemoglobin, various enzymes, and membranes of red blood cell from oxidative damage. As a result of oxidative stress from the Cl-compounds such as ClO_2, ClO_2^-, and ClO_3^-, GSH is converted to oxidized glutathione (GSSG). Chlorate decreased rat blood glutathione concentrations and

erythrocyte osmotic fragility. These changes were also observed with ClO_2 and ClO_2^- treatment (Abdel-Rahman et al., 1979).

The U.S. Environmental Protection Agency (EPA) currently recommends that the combined residues of ClO_2, ClO_2^-, and ClO_3^- not exceed 1.0 mg/L in the drinking water distribution system. To comply with such restrictions, typical dosages for treating drinking water are in the 0.04-0.4 mg/L range of ClO_2.

Chronic administration of ClO_2 and chlorate to rats in their drinking water did not produce any observed toxic effects at doses up to 10 mg/L. However, higher mortality rates were observed in rats administered 100 mg/L for 2 years (Hagg, 1949). Chlorite has been shown to be a potent stressor of blood capable of producing methemoglobinemia and hemolytic anemia both *in vivo* and *in vitro* (Heffernan et al., 1979). Chlorite and chlorate, but not chlorine dioxide, have been reported to produce hematological effects in nonhuman primates (Bercz et al., 1982). Chlorine dioxide, on the other hand, caused a decrease in serum thyroxin levels in monkeys. The hypothyroid effects were unique to ClO_2 since its end products, chlorite and chlorate, did not show this effect even at higher doses (Bercz et al., 1982). Other studies showed no significant decreases in serum thyroxin levels in adult rats fed up to 100 mg/L ClO_2 (Condie, 1986). However, administration of similar doses to pregnant and lactating females resulted in decreased serum thyroxin concentrations in rat pups (Condie and Bercz, 1985).

With regard to human studies, oral administration of ClO_2, chlorate and chlorite to healthy adult male volunteers did not produce any detrimental clinical effects. It is not

known whether long-term low-dose exposure to any of these chemicals will induce any severe adverse physiological effects.

Major Uses of Chlorine Dioxide

Water for poultry processing plants

Several studies were conducted to test the efficacy of ClO_2 treatment on controlling *Salmonella* spp. and other bacterial contamination in chiller water and on chicken broiler carcasses, and on extending the shelf-life and the sensory attributes of broiler carcasses. Chlorine dioxide has been reported to be a more effective bactericidal agent than HOCl in poultry processing (Lillard, 1980). A slow releasing ClO_2 solution (SRCD, Alcide) was added to turkey rinse and/or chilling water to reduce the incidence of *Salmonellae*. The in-plant chlorination system reduced the incidence of *Salmonellae* contaminated carcasses from an average of 70% after evisceration to 25% after chilling (Villarreal et al., 1990).

Commercially processed turkeys were chilled in rocker chillers containing ClO_2 as a bactericide. The microbial reduction on the finished birds was compared with that on birds chilled using the existing in-plant chlorination program. When contact times of 20-72 hours were used, ClO_2 treatment was most effective in reducing total and coliform counts on surface skin (Baran et al., 1973). Chiller water in a commercial broiler processing plant was treated with 20 ppm Cl_2, 34 ppm Cl_2, 3 ppm ClO_2, or 5 ppm ClO_2. Microbiologically, water samples were significantly better when 34 ppm Cl_2 or 5 ppm ClO_2 was used than 20 ppm Cl_2 or 3 ppm ClO_2. Despite differences in chill-water quality, all treatments significantly reduced bacterial counts on carcasses than those chilled in untreated water (Lillard, 1980). Shelf-life was prolonged for broiler carcasses treated with 1.33 and 1.39 mg/L ClO_2

compared to control carcasses. Sensory panelists reported no off-flavors for any of ClO_2-treated samples at all concentrations, but rated ClO_2-treated broiler skin as being slightly lighter in color compared to control carcasses (Thiessen et al., 1984).

Washing water for fruit and vegetables in food processing plants

Alternatives to hypochlorous acid (HOCl) are needed for the treatment of fruit and fruit-handling facilities. Chlorine dioxide was evaluated and found effective against common postharvest decay fungi, and against filamentous fungi occurring on fruit packinghouse surfaces. *In vitro* tests with conidial or sporangiospore suspensions of *Botrytis cinerea, Penicillium expansum, Mucor piriformis,* and *Cryptosporiopsis perennans* demonstrated >99% spore mortality within 1 min when the fungi were exposed to aqueous ClO_2 at 3 or 5 μg/mL (Roberts and Reymond, 1994). Longer exposure times were necessary for achieving similar spore mortalities with 1 μg/mL. Of the fungi tested, *B. cinerea* and *P. expansum* were the least sensitive to ClO_2. In comparison with untreated control, the number of filamentous fungi recovered was significantly lower in swipe tests from hard surfaces, such as belts and pads, in a commercial apple and pear packinghouse after treatment of surfaces with a 14 to 18 μg/mL ClO_2 foam formulation. Chlorine dioxide has the properties of a sanitizing agent for postharvest decay management when residues of postharvest fungicides are not desired or allowed (Roberts and Reymond, 1994). Control of postharvest diseases of tree fruits, especially apples, pears, and cherries, is becoming increasingly difficult. With the loss of several effective fungicides and decreasing residue tolerance for postharvest fungicides that remain, sanitation of both fruit and environmental surfaces must take a more prominent position as a disease management tool. Chlorine, usually as

hypochlorous acid, has long been the standard material of the fruit and vegetable industries for sanitation of process waters and fruit. A level of 100 μg/mL of free chlorine is currently recommended for control of postharvest pathogen spores in dump tanks and other recirculating water systems (Willett et al., 1989). Actual levels of free chlorine in process waters vary greatly from packinghouse to packinghouse.

The disadvantages of using chlorine include corrosion of metal equipment, reliance on manual monitoring of chlorine concentrations, sensitivity to organic load, effectiveness within a relatively narrow pH range, and formation of chlorinated by-products including chloroform (Hikada et al., 1992). The antimicrobial activity of ClO_2 in the presence of high levels of organic matter, such as those found in immersion dump tanks and flume processing waters, is not diminished as readily as that of chlorine (Benarde et al., 1965). The reported efficacy of ClO_2 against several postharvest pathogens (*in vitro* and *in vivo*) on pear fruit, against bacteria, and against protozoans warrants further investigation. Chlorine dioxide should be tested for its disinfection potential for use in the tree fruit industry and for its suitability to serve as a component of an integrated disease management system. Shelf-life of tomatoes can be improved by treatment with appropriate content of ClO_2 followed by storage under an atmosphere with low oxygen content (Rahman, 1973). Chlorine dioxide was evaluated to prevent microorganism buildup in waters used for cucumber washing, and for extending cucumber storage periods. Chlorine dioxide was shown to be at least 10-fold more effective than chlorine in killing microbes in cucumber waters. The process feeding rates of 20-35 ppm/hr appeared adequate to maintain effective and detectable ClO_2 levels in washing and hydrocooler waters. Holding cucumbers in water containing a high ClO_2 level

of 100 ppm failed to reduce microbes in blended samples. Chlorine dioxide treatments are also known to have no influence on natural lactic acid fermentation, but to delay microbial growth during cucumber storage (Costilow et al., 1984).

Drinking water

Chlorine dioxide is a disinfectant used in drinking water treatment. It has some important advantages over chlorine with respect to water quality when used for disinfection of drinking water (no formation of THMs, no impairment of taste and no odor). It was found that ClO_2 consumption is related to the dissolved organic carbon content of the water and the reaction time. Water samples from a plant that applied ozonation and activated carbon filtration had a very low ClO_2 consumption (Wondergem and Van, 1991).

Chlorine dioxide, as used in the treatment of drinking water, is generally obtained from sodium chlorite using either acidification or oxidation with chlorine. Under appropriate conditions, ClO_2 solutions contain only slight amounts (< 5%) of chlorate as the reaction product. Therefore, the importance of chlorates is virtually negligible. In most cases, the reaction products of ClO_2 are likely to be reduced to chlorite. Although chlorite is toxic and can cause methemoglobinemia, it is important to note that only low levels of this compound appear in treated water. The reported LC_{50} of sodium chlorite in rats is 140 mg/kg body weight.

Chlorine dioxide is more efficient for the disinfection of clean water. Therefore, this reagent is usually applied at an advanced stage of water treatment, as a postdisinfection compound for treated waters or as a preventive disinfectant for untreated groundwaters. One

of the other main advantages of using ClO_2 is that it does not react with ammonia. Consequently, it can be used when the ammonium content in the water is significant.

Current and future trends

Table 3 shows the concentrations of chlorine and ClO_2 allowed for direct use in food products. Chlorine dioxide at 5 ppm can be used in a sanitizing rinse for uncut, unpeeled fruits and vegetables. Potatoes (cut and peeled) can be rinsed with 1 ppm ClO_2. The National Food Processors Association (NFPA) has petitioned the Food and Drug Administration for allowance to use ClO_2-treated water to rinse cut or peeled fruits and vegetables (Food Chemical News, 1994). Currently, the FDA allows the use of ClO_2 as a disinfectant in rinse water that comes into contact with whole, unpeeled fruits and vegetables, provided the final product is rinsed with potable water. In its food additive petition to the FDA, NFPA asked the agency to extend its approval to include cut or peeled produce, and to eliminate the potable water rinse requirement when ClO_2 is used at 5 ppm. To support the petition, NFPA conducted research evaluating the safety of ClO_2 following contact with the cut produce.

The food industry's interest in ClO_2 is being driven by a desire to find alternatives to chlorine as a water disinfectant. Unlike chlorine, ClO_2 does not generate THMs from organic compounds. It offers comparable or superior bactericidal activity and forms more stable residues in water. The instability of pure, concentrated ClO_2 means the compound must be generated on site. This practice eliminates the risk associated with the transfer of gaseous chlorine. The NFPA research looked at whether use of ClO_2 on cut fruits and vegetables produced trihalomethanes or residues of chlorate and chlorite. These issues are important

Table 3. Direct food uses of chlorine and chlorine dioxide as approved by the FDA

	Food	Level (ppm)
Chlorine		
	Flour (bleaching)	1200 - 2500
	Fruit and vegetable	5-450
	Fish processing and thawing	5-10
	Thawing of frog legs	200-500
	Whole grains	150
	Poultry giblets (livers, hearts)	35
	Poultry processing (fresh poultry)	50
Chlorine dioxide		
	Fruit and vegetable (uncut)	5
	Potatoes (cut and peeled)	1
	Poultry processing	3

because it is unknown whether these harmful compounds can form at the cut surfaces of produce under low concentrations of ClO_2.

The FDA announced on March 3, 1995 the clearance of a food additive petition allowing the use of 3 ppm residual ClO_2 to control the microbial population in process waters contacting whole poultry carcasses (FDA, 1995). Chlorine dioxide is currently used as a component of a sanitizer solution, a bleaching agent for flour, and is approved by the EPA for use in potable water treatment plants.

The FDA replaced the term "disinfect" which implies total eradication of microbial contamination. FDA has concluded that "no detectable residues of ClO_2" would remain on

exposed carcasses. Exposure to chlorite and chlorate as a result of this use would be "virtually nil". The agency evaluated information on levels of oxidation-sensitive fatty acids such as oleic, linoleic and arachidonic acid, in raw untreated poultry as well as in poultry exposed to process water containing ClO_2. The agency concluded that exposure to levels "7 to 10" times higher than prescribed did not result in "appreciable loss" of these acids. The agency also concluded that "the use of ClO_2 in process water should not pose a significant health concern from the formation of mutagenic substances" (FDA, 1995).

The FDA also considered the potential oxidative effects of ClO_2, chlorite, and chlorate on poultry. When the information on thiobarbituric acid (TBA) values of raw and cooked poultry exposed to ClO_2-containing process water was evaluated; these samples showed no difference in TBA values from that for poultry exposed to tap water (Food Chemical News, 1995). The TBA test is commonly used as an indication of oxidative decomposition (and of rancidity) in meat and fat; higher TBA values indicate more oxidative decomposition.

CHAPTER III
DETERMINATION OF THE EFFECTIVENESS OF CHLORINE DIOXIDE IN CONTROLLING BACTERIAL LOADS AND QUALITY OF VARIOUS SEAFOOD PRODUCTS OVER A 7-DAY STORAGE PERIOD

Introduction

Chlorine dioxide (ClO_2) is widely used in Europe as an alternative to chlorine for drinking water disinfection (Symons et al., 1978). It is currently used in approximately 500 drinking water treatment plants in the U.S., including such cities as Philadelphia, Shreveport, El Paso, and Galveston. Chlorine dioxide is soluble in water, and does not react with ammonia like chlorine. It has a greater oxidizing capacity than chlorine. Its lethality towards bacteria is not affected by high pH. The bactericidal mechanism of ClO_2 was believed to involve the loss of permeability control with nonspecific oxidative damage to the outer membrane and subsequent destruction of the transmembrane ionic gradient (Berg et al., 1986). As a bactericide, ClO_2 was tested to reduce bacterial numbers in poultry chiller water (Tsai et al., 1995), on fecally contaminated beef carcass (Cutter and Dorsa, 1995), and in cucumber hydrocooling water (Reina et al., 1995). Chlorine dioxide treatments extended the shelf-life of broilers (Lillard, 1980) and reduced the incidence of *Salmonella* spp. on poultry carcasses (Thiessen et al., 1984). However, there are no reports on the use of ClO_2 as a sanitizer for seafood treatment.

Therefore, to help determine the potential usage of ClO_2 in the seafood industry, fillets of Atlantic salmon (*Salmo salar*) and red grouper (*Epinephelus morio*), headless brown shrimp (*Penaeus aztecus*), calico scallops (*Aequipecten gibbus*), and gutted whole Atlantic salmon and red grouper were treated with freshly prepared aqueous ClO_2 solutions at 20, 40, 100, and 200 ppm total available ClO_2 in 3.5% brine for 5 min. Bacterial loads and sensory quality were evaluated on day 0, 3 and 7 of cold storage on ice. Triplicate runs were tested for each sample at each treatment condition and the experiment was repeated at least once. The main objective was to determine if treatment with ClO_2 will improve the quality of seafood products by extending their shelf-life.

Materials and Methods

Chlorine-demand-free water

Chlorine-demand-free water (CDF water) was prepared following the method of Ghanbari et al. (1982) by passing distilled water through two successive Barnstead deionizing units (Barnstead Inc., Dubuque, IA) and then a glass column containing Porapak® Q (Supelco, Inc.). This water was used to prepare all reagents.

Preparation of stock chlorine dioxide and working solutions

A mixture of 2% ClO_2 and 98% inert ingredients (Oxine® concentrate, OC) was supplied by Bio-Cide International Inc. (Norman, OK). A stock solution containing 2,000 ppm available ClO_2 was freshly prepared by first reacting OC for 5 min at room temperature with 85% phosphoric acid at a 20:1 (v/v) ratio in a brown flask sealed with a glass stopper and then diluting the reaction mixture with 9 volumes of ice-cold CDF water. This stock solution was used to prepare various working solutions (20, 40, 100 and 200 ppm) in 3.5%

ice-cold brine. Brine was prepared by dissolving 35 g NaCl in 1 L CDF water. Both the stock and working ClO_2 solutions were freshly prepared on the day of experiment.

Determination of the bactericidal effectiveness of ClO_2 solution in treated red grouper and salmon fillets, calico scallops, brown shrimp, and whole red grouper and salmon

Whole (gutted) fish of salmon and red grouper, as well as their fillets were purchased from a local seafood store. Calico scallops packed by Seasweet Scallop Company (Cape Canaveral, FL) and brown shrimp (headless and shell on, 38 tails/lb) from Jubilee Foods Inc. (Bayou La Batre, AL) were obtained through the assistance of Dr. Steve Otwell of the Food Science and Human Nutrition Department, University of Florida. The shrimp sample reportedly received no previous sulfite or phosphate treatment.

Preparation and bacterial enumeration of ClO_2-treated fish fillets. The fillets of red grouper and salmon were cut into pieces of about 50 g each. After 54 pieces of red grouper and salmon fillets were weighed, they were dipped in an equal volume (1:1, w/v) of ice-cold sterile brine for 1 min. Following the draining off of extra liquid, 9 pieces were randomly chosen and each treated with 4 volumes (1:4, w/v) of ice-cold sterile brine (control) or freshly prepared ClO_2 solutions at 20, 40, 100 or 200 ppm in brine for 5 min with continuous stirring. An adequate amount of 1 N $Na_2S_2O_3$ solution was added to quench residual chlorine. After excess liquid was drained off, each piece was placed in separate Whirl-Pak® bags (Nasco, Fort Atkinson, WI). Each bag was properly labeled and then stored in a styrofoam box filled with crushed ice for 7 days in a 5 °C walk-in cold room. The pH of the test solutions before and after dipping the fish fillets were also monitored. The experiments

with red grouper and salmon fillets were repeated once. The remaining 9 pieces served as the non-treated control.

On each day of testing (day 0, 3, and 7), 3 pieces of red grouper or salmon were removed from each treatment group. Bacterial enumeration was performed by adding nine volumes (1:9, w/v) of sterile peptone water (0.1%) to each fish sample in a sterile Whirl-Pak® bag. The bag was placed between the palms of the hands and pressed while rubbing the bag backwards and forwards. After pressing and rubbing the bag by hand for 2 min, the peptone water was removed and serially diluted with sterile Butterfield's buffer. Each diluent was then surface plated on quadruplicate PCA plates containing 1.5% NaCl. Pour plate method was also used for some samples. Bacterial colonies on the plates were counted after incubation at 25 °C for 72 hrs.

Preparation and bacterial enumeration of ClO_2-treated calico scallops and brown shrimp. Approximately 1800 g of scallops or shrimp were dipped in an equal volume (1:1, w/v) of ice-cold sterile brine for 1 min. Extra liquid was drained off and portions of approximately 100 g were removed and randomly treated in 2 L beakers with 4 volumes (1:4, w/v) of ice-cold sterile brine or ClO_2 solution (20, 40, 100, and 200 ppm TACD) in brine. After the extra liquid was drained off, 3 portions (about 30 g each of treated sample) were removed from each group and placed in separate Whirl-Pak® bags. An additional 9 bags (about 30 g each) of nontreated scallops or shrimp were also prepared to serve as controls. This experiment was repeated once.

On each day of testing (day 0, 3, and 7), 3 bags were removed from each treatment group. Bacterial enumeration was performed by homogenizing at high speed for 2 min the

samples with nine volumes of 0.1% peptone water (1:9, w/v) in sterile blender jars. The homogenates were serially diluted with sterile Butterfield's buffer and the diluents then surface plated on quadruplicate PCA plates containing 1.5% NaCl. Pour plate method was also used for some samples. Bacterial colonies on the plates were counted following incubation of the plates at 25 °C for 72 hrs. The pH of the test solutions before and after dipping the scallops or shrimp was also monitored.

Preparation and bacterial enumeration of ClO_2-treated whole fish. Eighteen salmon (about 3.6 kg each) or red grouper (about 22 inches) were washed under running water for 2 minutes. The fishes were treated in a clean container with 4 volumes (1:4, w/v) of ice-cold sterile brine or ClO_2 solution (20, 40, 100, and 200 ppm TACD) in brine. After the extra liquid was drained off, each fish was labeled and then displayed on a stainless steel table for sensory evaluation. Three fish was used for each treatment group. The nontreated control group also had 3 fish. The samples were stored for 7 days in a seafood display chamber (3 °C) with crushed ice.

The bacterial numbers on the whole fish were determined by removing two skin areas each 3 x 3 cm^2 from each fish using a sterile surgical blade and forceps. For salmon, two additional muscle areas each 3 x 3 cm^2 were removed from inside the abdominal cavity of each fish. After the two test areas (the skin or muscle) from the same fish were removed, they were put into sterile Whirl-Pak® bags and weighed. To the bags were added nine volumes (1:9, w/v) of sterile 0.1% peptone water. The sample bags were then subjected to rubbing by hand for 2 min. The peptone water was removed and serially diluted with sterile Butterfield's buffer for bacterial enumeration using surface plating or pour plate methods.

On day 3 and 7 of storage, the skin (and muscle also for salmon) samples were removed again from each fish for determination of time-related changes of bacterial numbers. Since each test fish was labeled individually, the changes in bacterial numbers on each individual fish over the 7-day period could be determined. The experiment with red grouper was repeated once, while the test with salmon was repeated twice.

Sensory evaluation of test seafood samples

On each day of testing (day 0, 3, and 7), the seafood samples from each treatment group were prepared for quality evaluation. Ten experienced panelists wearing gloves were requested to evaluate the quality of the control and treated seafood (fillets of red grouper and salmon, calico scallops, brown shrimp, and whole red grouper and salmon) to determine whether differences occurred among the samples. At a preliminary training session, panelists were given various seafood treatments which included fresh, stale, and excessive ClO_2 treated seafood in order to establish a fairly uniform degree of sensory evaluation. Panelists also used descriptive analysis to identify the characteristics that distinguished the samples and rated intensity of important sensory attributes. The protocols and evaluations described in the National Marine Fisheries Service (NMFS, 1975) Fishery Products Inspection Manual were followed.

Tables A-1 through A-6 in Appendix are the evaluation sheets used by the panelists to assess and record the quality of each test sample (A1, A2, and A3) under each treatment condition (A-F). Items included for sensory evaluation were appearance defects, discoloration and formation of odor for tested fish fillets (Table A-1), discoloration and formation of odor for scallops and shrimp (Table A-2), and appearance defects, skin

discoloration, eye color, body damage, gill and gut cavity, belly flaps, surface defects and formation of odor for tested whole red grouper and salmon (Table A-3). Triplicate samples were used for each treatment group. Except for the test with salmon, experiments with the seafood were conducted twice. The tests with salmon were conducted three times. The degree of abnormality (or defect) was categorized into slight, moderate and excessive. Tables A-4, A-5 and A-6 are the score sheets used to assess the grade (A, B, and C) of each test sample under each treatment condition over the 7 days of storage.

According to the Regulations Governing Processed Fishery Products and U.S. Standards for Grades of Fishery Products (NMFS, 1975), U.S. Grade A fish fillets shall (1) possess good flavor and odor characteristic of the species, and (2) comply with the limits for defects for U. S. Grade A quality as outlined in §263.104. U.S. Grade B fish fillets shall (1) possess reasonably good flavor and odor characteristic of the species, and (2) comply with the limits for defects for U. S. Grade B quality in accordance with §263.104. U.S. Grade C fish fillets shall (1) possess minimal acceptable flavor and odor characteristic of the species with no objectionable off-flavors or off-odors, and (2) comply with the limits for defects for U. S. Grade C quality in accordance with §263.104.

U.S. Grade A shrimp shall (1) possess good flavor and odor characteristics of the species as defined in §265.104(d), and (2) not exceed the tolerance level for defects for U.S. Grade A quality set forth in §265.104 Table II. U.S. Grade B shrimp shall (1) possess reasonably good flavor and odor characteristics of the species as defined in §265.104(d), and (2) not exceed the tolerance level for defects for U.S. Grade B quality set forth in §265.104 Table II. U.S. Grade C shrimp shall (1) possess minimum acceptable flavor and odor

characteristics of the species as defined in §265.104(d), and (2) not exceed the tolerance level for defects for U.S. Grade C quality set forth in §265.104 Table II.

U.S. Grade A whole fish shall (1) possess good flavor and odor, and (2) comply with the limits for defects for U. S. Grade A quality in accordance with §261.104. U.S. Grade B whole fish shall (1) possess reasonably good flavor and odor, and (2) comply with the limits for defects for U. S. Grade B quality in accordance with §261.104. U.S. Substandard whole fish does not possess reasonably good flavor and odor and/or exceeds the limits for defects for U. S. Grade B quality in accordance with §261.104.

Statistical analysis

Bacterial numbers in CFU/g sample were transformed to $\log_{10}$ for statistical analysis. An analysis of variance (ANOVA) was performed by SAS using the general linear models procedure (SAS Institute, Inc. 1989). Duncan's multiple range test was used to obtain pairwise comparisons among sample means. Evaluations were based on a P=0.05 significance level.

Results and Discussion

Table 4 shows that the concentration of ClO_2 in brine caused a dose-related decrease in pH values. Dipping of red grouper or salmon fillets, scallops and shrimp caused an increase in pH values in both the treated brine and ClO_2 solutions. The bactericidal effectiveness of ClO_2 was well established after evaluating the treated ClO_2 solutions. Microflora were not detected in ClO_2 solutions at 100 and 200 ppm after treatment. Low bacterial loads were detected only in the treated 20 ppm or 40 ppm ClO_2 solutions (Table 4). A rusty color was noticed with red grouper fillets and scallops following treatment with

Table 4. Changes in pH values of test solutions following treatment of seafood samples and determination of bacterial numbers in treated solutions

	Experiment #1			Experiment #2		
	pH		Bacterial number (CFU/mL)	pH		Bacterial number (CFU/mL)
	Before treatment[c]	After treatment[d]		Before treatment[c]	After treatment[d]	
Red grouper fillets						
Brine[a]	5.44	6.19	4.93×10^4	5.66	6.15	9.60×10^3
Treated brine[b]	5.40	6.04	5.73×10^3	5.66	6.10	1.10×10^3
20 ppm ClO_2	3.70	5.00	8.30×10^1	3.73	5.13	3.30×10^2
40 ppm	3.41	4.58	0	3.44	4.47	0
100 ppm	3.08	3.93	0	3.06	3.81	0
200 ppm	2.85	3.23	0	2.71	3.07	0
Salmon fillets						
Brine[a]	6.10	6.21	1.49×10^4	6.16	6.24	2.38×10^3
Treated brine[b]	6.10	6.13	9.40×10^2	6.16	6.04	1.31×10^2
20 ppm ClO_2	3.74	4.53	3.30×10^1	3.87	4.59	7
40 ppm	3.30	3.95	0	3.53	4.04	0
100 ppm	2.93	3.33	0	3.14	3.48	0
200 ppm	2.73	2.99	0	2.74	3.03	0

[a] Brine means that it used as a prewashing solution on all seafood. The seafood was dipped in equal volume (1:1, w/v) of ice-cold sterile brine for 1 min

[b] Treated brine means it was used as a treatment control on seafood in increments of 4 volumes (1:4, w/v) of ice-cold sterile brine for 5 min

[c] Before treatment means pH of brine or ClO_2 working solution before seafood treatment

[d] After treatment means pH of brine or ClO_2 working solution after seafood treatment

Table 4--Continued

	Experiment #1			Experiment #2		
	pH		Bacterial number (CFU/mL)	pH		Bacterial number (CFU/mL)
	Before treatment[c]	After treatment[d]		Before treatment[c]	After treatment[d]	
Calico scallops						
Brine[a]	6.25	ND[1]	3.77×10^4	6.19	ND[1]	3.10×10^4
Treated brine[b]	5.45	6.19	2.28×10^3	6.14	6.09	3.35×10^3
20 ppm ClO_2	3.87	5.77	3.70×10^2	3.68	5.64	8.40×10^2
40 ppm	3.58	5.42	7.40×10^1	3.35	5.16	1.40×10^1
100 ppm	3.26	4.77	0	2.98	4.32	0
200 ppm	2.99	3.98	0	2.73	3.56	0
Brown shrimp						
Brine[a]	6.10	ND[1]	5.80×10^4	6.21	ND[1]	1.53×10^5
Treated brine[b]	6.10	7.43	9.50×10^3	6.12	6.79	1.37×10^4
20 ppm ClO_2	3.74	5.72	4.30×10^2	4.02	5.62	4.20×10^2
40 ppm	3.30	4.59	0	3.49	4.47	1.06×10^2
100 ppm	2.93	3.44	0	3.07	3.61	0
200 ppm	2.73	2.91	0	2.80	3.18	0

[1] Not determined

[a] Brine means that it used as a prewashing solution on all seafood. The seafood was dipped in equal volume (1:1, w/v) of ice-cold sterile brine for 1 min

[b] Treated brine means it was used as a treatment control on seafood in increments of 4 volumes (1:4, w/v) of ice-cold sterile brine for 5 min.

[c] Before treatment means pH of brine or ClO_2 working solution before seafood treatment

[d] After treatment means pH of brine or ClO_2 working solution after seafood treatment

Table 4--Continued

	Experiment #1			Experiment #2		
	pH		Bacterial number (CFU/mL)	pH		Bacterial number (CFU/mL)
	Before treatment[c]	After treatment[d]		Before treatment[c]	After treatment[d]	
Whole red grouper[a]						
Treated brine[b]	ND[1]	6.2	1.64×10^4	5.99	6.18	1.20×10^4
20 ppm ClO_2	3.6	4.01	0	3.63	4.01	0
40 ppm	3.29	3.47	0	3.18	3.34	0
100 ppm	2.93	3.12	0	2.96	3.1	0
200 ppm	2.67	2.8	0	2.61	2.74	0
Whole salmon[a]						
Treated brine[a]	6.07	6.13	1.27×10^2	6.01	6.18	1.33×10^4
20 ppm ClO_2	3.69	4.04	0	3.61	4.15	0
40 ppm	3.29	3.6	0	3.31	3.52	0
100 ppm	2.96	3.09	0	2.98	3.14	0
200 ppm	2.73	2.72	0	2.71	2.83	0

[1] Not determined
[a] All fish samples were prewashed with running tap water for 2 min
[b] Treated brine means it was used as a treatment control. The fish were treated with 4 volumes (1:4, w/v) of ice-cold sterile brine for 5 min
[c] Before treatment means pH of brine or ClO_2 working solution before seafood treatment
[d] After treatment means pH of brine or ClO_2 working solution after seafood treatment

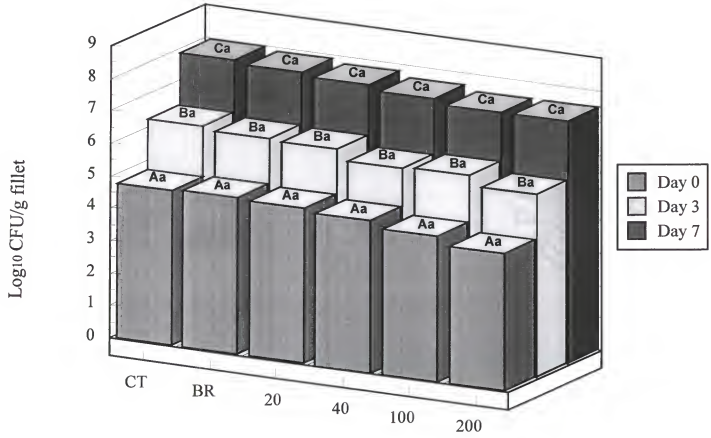
100 or 200 ppm ClO_2 solution. Discoloration of salmon fillets also occurred after treatment with ClO_2 solutions at the same concentrations.

Fish fillets

Compared to the nontreated control and brine treated groups, treatment of red grouper fillets with ClO_2 solutions caused a dose-related decrease in numbers of natural microflora (Figure 3). However, the difference in bacterial numbers was not significantly ($P>0.05$) different among the six groups. The number of microflora in each group increased significantly following storage of the fillets for 3 or 7 days. The treated red grouper fillets with ClO_2 solutions at 40, 100 and 200 ppm had lower bacterial numbers than the other three groups following storage for 3 and 7 days.

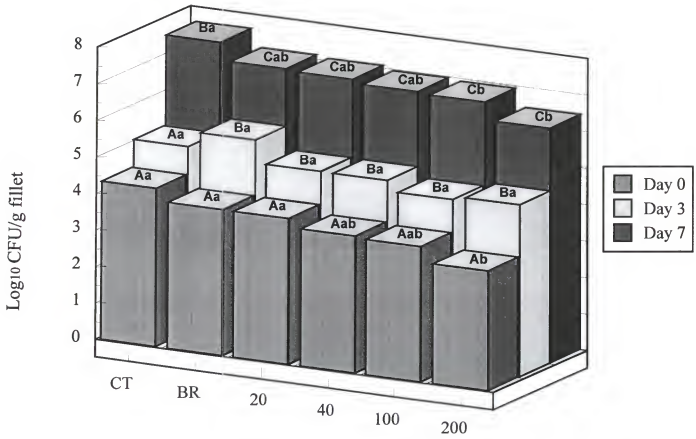
The treatment of salmon fillets with ClO_2 solution on day-0 caused a dose-related decrease in natural microflora (Figure 4). Except for the 200 ppm ClO_2 group, the difference in bacterial number was not significant ($P>0.05$) among the other five groups. This dose-related effect on bacterial numbers by ClO_2 was also noticed for treated samples stored for 3 or 7 days. Salmon fillets treated with 100 or 200 ppm ClO_2 had significantly ($P<0.05$) less bacterial numbers than the nontreated control on day-7 of storage.

Nontreated fillets of red grouper and salmon and those treated with brine and ClO_2 solutions at 20 or 40 ppm usually had very good quality (Grade A) showing no appearance defects or discoloration (Tables 5 and 6). No fishy odor was noticed in any of the test fillets used on day 0. However, for the red grouper, the nontreated and brine controls, and 20 ppm ClO_2 started to show problems with flesh consistency and discoloration following cold storage for 3 days and continued to worsen up to the 7 day storage. In addition, a fishy smell



A,B,C - Within each day, means followed by the same letter are not significantly different from each other at P=0.05
a,b,c - Within each treatment, means followed by the same letter are not significantly different from each other at P=0.05
CT = control, BR = brine

Figure 3. Time-related changes in bacterial numbers on red grouper fillets following treatment with chlorine dioxide solutions at different concentrations (ppm)



A,B,C - Within each day, means followed by the same letter are not significantly different from each other at P=0.05
a,b,c - Within each treatment, means followed by the same letter are not significantly different from each other at P=0.05
CT = control, BR = brine

Figure 4. Time-related changes in bacterial numbers on salmon fillets following treatment with chlorine dioxide solutions at different concentrations (ppm)

Table 5. Grading of red grouper fillets following treatment with ClO_2 solutions or brine and then storage at 4 °C for 0, 3 or 7 days

Treatment	Day	Experiment #1			Experiment #2		
		Replicate			Replicate		
		I	II	III	I	II	III
Control	0	A	A	A	A	B	B
	3	A	A	A	B	B	B
	7	C	B	C	B	B	B
Brine	0	A	A	A	A	A	B
	3	A	B	B	B	B	B
	7	C	C	C	B	B	B
20 ppm	0	A	A	A	B	A	A
	3	A	A	B	B	A	B
	7	B	C	C	B	B	B
40 ppm	0	A	A	A	B	A	B
	3	A	A	A	B	A	B
	7	B	B	B	B	B	B
100 ppm	0	B	A	B	B	B	B
	3	B	B	B	B	B	B
	7	C	B	C	B	B	B
200 ppm	0	B	B	B	B	B	B
	3	C	B	C	B	B	B
	7	C	B	C	B	B	B

Table 6. Grading of salmon fillets following treatment with ClO_2 solutions or brine and then storage at 4 °C for 0, 3 or 7 days

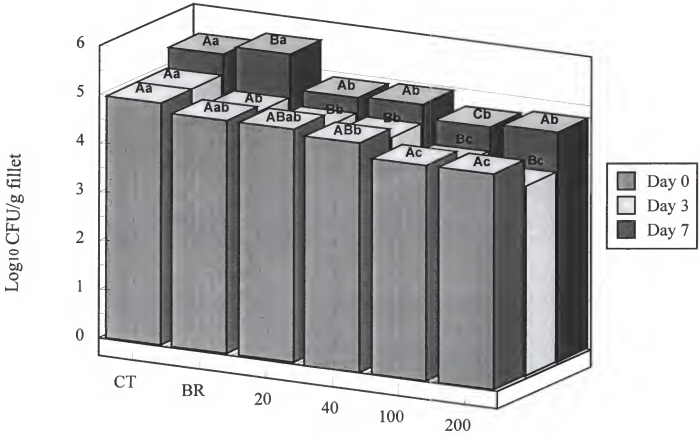
Treatment	Day	Experiment #1			Experiment #2		
		Replicate			Replicate		
		I	II	III	I	II	III
Control	0	A	A	A	A	A	A
	3	B	B	B	A	A	A
	7	C	C	B	A	A	B
Brine	0	A	A	A	A	A	A
	3	A	A	A	B	A	A
	7	B	B	A	B	B	B
20 ppm	0	A	A	A	A	A	A
	3	B	B	B	A	A	B
	7	B	B	B	A	B	B
40 ppm	0	A	A	A	A	A	A
	3	B	A	A	A	A	A
	7	B	A	B	A	A	A
100 ppm	0	A	A	B	B	B	B
	3	B	B	B	B	B	B
	7	B	B	B	B	B	B
200 ppm	0	B	B	B	B	B	B
	3	B	B	B	B	B	B
	7	B	C	B	B	B	B

occurred with some of the test fillets. Similar observations were noticed with nontreated salmon fillets. Salmon fillets treated with brine or ClO_2 solutions at 20 or 40 ppm seemed to have better quality than nontreated controls.

Calico scallops

Compared to the nontreated control group on day 0, scallops treated with ClO_2 showed a dose-related decrease in the number of natural microflora (Figure 5). The groups treated with ClO_2 solutions at 40, 100 and 200 ppm had significantly ($P<0.05$) less bacterial numbers than the nontreated control. At day 3, those scallops treated with ClO_2 solutions at 100 and 200 ppm had significantly ($P<0.05$) less bacterial numbers than the brine and the 20 and 40 ppm treated groups, which in turn had significantly ($P<0.05$) less bacterial numbers than the untreated control. Compared to day 0, those groups treated with 100 and 200 ppm ClO_2 had significantly less bacterial numbers following three days of storage. The bacterial numbers then increased for the extended 7 days storage. However, the groups treated with ClO_2 solutions still had significantly less bacterial numbers than the nontreated and brine treated control groups.

A fishy odor rather than a seaweed odor occurred with nontreated calico scallops and contributed to their initial Grade B evaluation (Table 7). However, treatment of the scallops with brine or ClO_2 solutions at 20 or 40 ppm appeared to remove the odor and provided the basis for better scoring of the treated scallops (data not shown). Scallops treated with ClO_2 solutions at 100 or 200 ppm had an unfavorable rusty color. The presence of the rusty color together with the formation of odor contributed to the lower grading for these scallops following cold storage for 7 days (Table 7).



A,B,C - Within each day, means followed by the same letter are not significantly different from each other at P=0.05
a,b,c - Within each treatment, means followed by the same letter are not significantly different from each other at P=0.05
CT = control, BR = brine

Figure 5. Time-related changes in bacterial numbers on calico scallops following treatment with chlorine dioxide solutions at different concentrations (ppm)

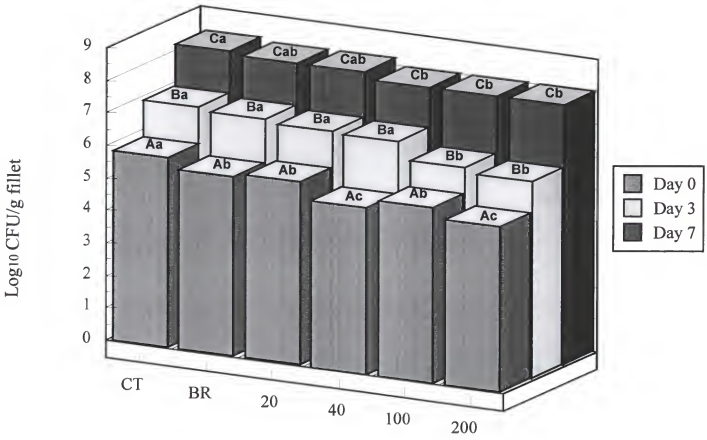
Table 7. Grading of calico scallops following treatment with ClO_2 solutions or brine and then storage at 4 °C for 0, 3 or 7 days

Treatment	Day	Experiment #1			Experiment #2		
		Replicate			Replicate		
		I	II	III	I	II	III
Control	0	B	B	A	B	B	B
	3	B	B	B	C	B	B
	7	B	C	C	C	B	B
Brine	0	A	A	A	A	B	B
	3	A	A	A	B	B	B
	7	B	B	B	B	B	B
20 ppm	0	A	A	A	B	A	A
	3	A	A	A	B	A	A
	7	C	B	B	B	A	B
40 ppm	0	A	A	A	B	B	B
	3	B	A	B	B	B	B
	7	B	B	C	B	B	B
100 ppm	0	B	B	B	B	B	B
	3	B	B	B	B	B	B
	7	B	C	C	C	C	B
200 ppm	0	B	B	B	B	C	B
	3	B	B	B	B	C	B
	7	B	C	B	B	C	C

The quality of nontreated scallops deteriorated following cold storage for 3 or 7 days due to discoloration and odor formation. Those scallops treated with brine or ClO_2 solutions at 20 or 40 ppm maintained a better quality than the nontreated control after cold storage for 3 or 7 days (Table 7). Odor formation and discoloration also occurred but to a lesser extent than the nontreated control.

Brown shrimp

The number of natural microflora in each group increased significantly ($P<0.05$) following storage of shrimp at 4 °C for 3 or 7 days. Compared to the nontreated control group, shrimp treated with ClO_2 solutions on day-0 caused a dose-related and significant ($P<0.05$) decrease in numbers of natural microflora (Figure 6). Shrimp samples treated with ClO_2 solutions at 100 or 200 ppm had significantly less bacterial numbers than the other four groups following 3 days of storage. This dose-related bactericidal effect of ClO_2 was also noticed for the day 7 samples. Compared to nontreated brown shrimp and treated ClO_2 solutions at 20 or 40 ppm on the day of testing, shrimp treated with 100 or 200 ppm ClO_2 solutions had slight discoloration (melanosis, not the rusty color as in the case of treated scallops or treated grouper fillets, due to the presence of the shrimp shell) and the presence of a chlorine smell. The blackening worsened for treated samples with ClO_2 solutions at 100 or 200 ppm following cold storage for 3 or 7 days. Melanosis and odor formation also occurred with nontreated shrimp and those treated with ClO_2 solutions at 20 or 40 ppm following cold storage for 3 or 7 days. Table 8 shows the grading of brown shrimp following the various treatments.



A,B,C - Within each day, means followed by the same letter are not significantly different from each other at P=0.05
a,b,c - Within each treatment, means followed by the same letter are not significantly different from each other at P=0.05
CT = control, BR = brine

Figure 6. Time-related changes in bacterial numbers on brown shrimp following treatment with chlorine dioxide solutions at different concentrations (ppm)

Table 8. Grading of brown shrimp following treatment with ClO_2 solutions or brine and then storage at 4 °C for 0, 3 or 7 days

Treatment	Day	Experiment #1			Experiment #2		
		Replicate			Replicate		
		I	II	III	I	II	III
Control	0	A	A	A	B	B	B
	3	B	A	B	B	B	B
	7	B	B	B	B	B	C
Brine	0	A	A	A	A	A	A
	3	B	A	B	B	B	B
	7	B	A	B	B	B	B
20 ppm	0	B	A	B	B	B	B
	3	B	A	B	B	B	B
	7	B	B	B	B	B	B
40 ppm	0	A	B	A	A	B	B
	3	A	B	A	B	B	B
	7	B	B	B	B	B	B
100 ppm	0	A	B	B	B	A	B
	3	A	B	B	B	B	B
	7	B	B	B	B	B	B
200 ppm	0	B	B	B	B	B	B
	3	B	B	B	B	B	B
	7	B	B	C	B	C	B

In a separate trial, brown shrimp with a bacterial load of 5.87 $\log_{10}$ CFU/g were treated with ClO_2 solutions at 20, 40, 100 or 200 ppm and then stored at -20 °C for 1 week. Compared to the nontreated control, shrimp treated with ClO_2 showed a dose-related decrease in bacterial numbers following storage for 3 and 7 days. (See the table below). Furthermore, extending storage of ClO_2-treated samples to 7 days caused a further reduction in bacterial numbers. Thus, treatment with ClO_2 solution followed by frozen storage at -20 °C seemed to effectively decrease bacterial loads on shrimp. Thus, the seafood industry may be able to treat shrimp with ClO_2 for initial control of bacterial loads and then use cold storage at -20 °C to further control microbial quality and extend the shelf-life of the product.

	Log_{10} CFU/g shrimp	
	day 3	day 7
nontreated control	5.74	5.61
20 ppm	5.39	5.23
40 ppm	5.42	4.98
100 ppm	5.32	4.86
200 ppm	4.75	4.52

Whole fish (grouper and salmon)

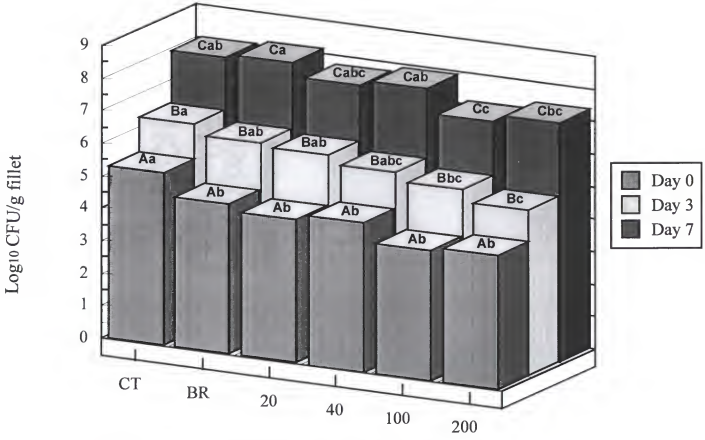
The bactericidal effectiveness of ClO_2 was well demonstrated. No bacteria were detected on fish treated with ClO_2 solutions at 100 and 200 ppm (Table 4). Variations in initial bacterial numbers occurred in the test trial. Furthermore, such variation in initial bacterial numbers also occurred with test fish from different batches used in separate trials.

Therefore, the bactericidal efficacy of ClO_2 was not clearly demonstrated when data from separate trials were pooled.

In most cases, red grouper treated with ClO_2 solutions at 100 or 200 ppm and then stored at 4° C for 3 or 7 days had significantly less bacterial numbers on the skin than nontreated control or brine treated samples (Figure 7). The natural microflora on nontreated and treated (with brine and ClO_2 solutions) salmon, as determined from the skin or muscle areas, increased significantly ($P<0.05$) following storage of the fish at 4 °C for 3 or 7 days (Figures 8 and 9). Salmon treated with ClO_2 solutions showed a dose-related decrease in bacterial numbers as determined from the skin areas. However, for natural microflora occurring in the abdominal cavity of salmon as determined from the muscle areas, the treatment with ClO_2 solutions caused a dose-related and significant ($P<0.05$) decrease in numbers. This same finding also occurred with treated salmon following storage at 4 °C for 3 or 7 days.

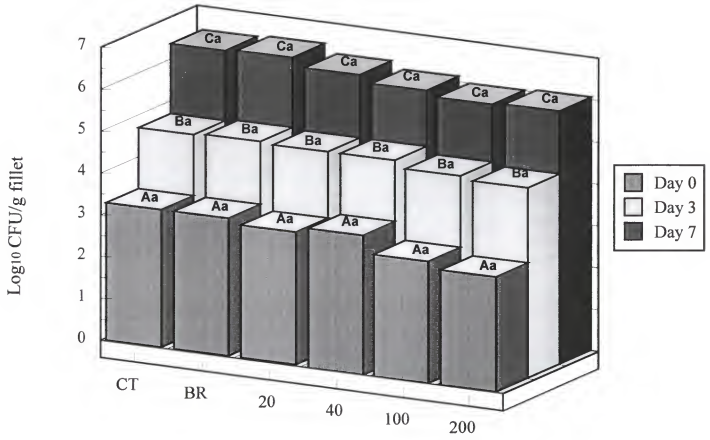
Treatment with ClO_2 solutions at 100 or 200 ppm caused discoloration (bleaching) of the skin surface of the whole red grouper and salmon. Additionally, the fishy odor disappeared from ClO_2-treated fish and a light chocolate color developed on the gills of the treated fish (Figure 10). The color development was related to the concentration of ClO_2 solutions used. The fish eyes also changed color due to treatment with ClO_2 solutions. Such a phenomena did not occur with nontreated or brine treated samples on day 0 of the experiment.

The sensory quality of ClO_2-treated red grouper and salmon was less favorable following storage at 4 °C for 3 or 7 days (Tables 9 and 10). Discoloration of the skin to an



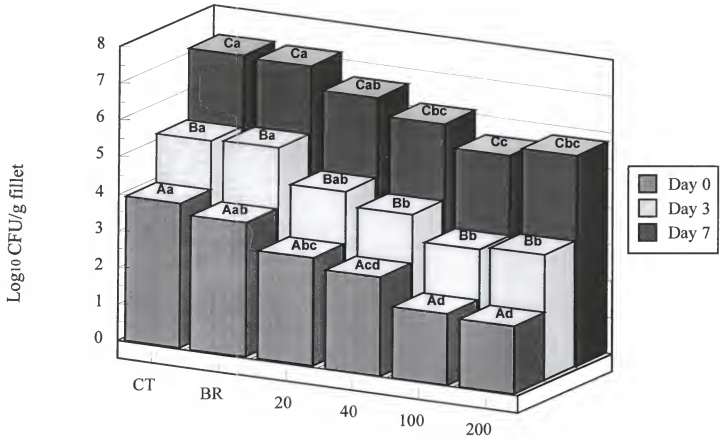
A,B,C - Within each day, means followed by the same letter are not significantly different from each other at P=0.05
a,b,c - Within each treatment, means followed by the same letter are not significantly different from each other at P=0.05
CT = control, BR = brine

Figure 7. Time-related changes in bacterial numbers on whole grouper (skin) following treatment with chlorine dioxide solutions at different concentrations (ppm)



A,B,C - Within each day, means followed by the same letter are not significantly different from each other at P=0.05
a,b,c - Within each treatment, means followed by the same letter are not significantly different from each other at P=0.05
CT = control, BR = brine

Figure 8. Time-related changes in bacterial numbers on whole salmon (skin) following treatment with chlorine dioxide solutions at different concentrations (ppm)



A,B,C - Within each day, means followed by the same letter are not significantly different from each other at P=0.05
a,b,c - Within each treatment, means followed by the same letter are not significantly different from each other at P=0.05
CT = control, BR = brine

Figure 9. Time-related changes in bacterial numbers on whole salmon (muscle) following treatment with chlorine dioxide solutions at different concentrations (ppm)

Brine treatment

200 ppm ClO_2 treatment

Figure 10. Change in gill color following treatment with brine (control) and a ClO_2 solution at 200 ppm

Table 9. Grading of whole red grouper following treatment with ClO_2 solutions or brine and then storage at 4 °C for 0, 3 or 7 days

Treatment	Day	Experiment #1			Experiment #2		
		Replicate			Replicate		
		I	II	III	I	II	III
Control	0	B	B	B	B	B	B
	3	B	[1]C	C	B	B	B
	7	B	C	C	B	B	B
Brine	0	B	B	B	B	B	B
	3	B	C	C	B	B	C
	7	B	C	C	B	B	C
20 ppm	0	B	C	C	C	B	C
	3	C	C	C	C	C	C
	7	C	C	C	C	C	C
40 ppm	0	C	C	C	C	C	C
	3	C	C	C	C	C	C
	7	C	C	C	C	C	C
100 ppm	0	C	C	C	C	C	C
	3	C	C	C	C	C	C
	7	C	C	C	C	C	C
200 ppm	0	C	C	C	C	C	C
	3	C	C	C	C	C	C
	7	C	C	C	C	C	C

[1]C means substandard.

U.S. substandard: whole fish does not possess reasonably good flavor and odor

Table 10. Grading of whole salmon following treatment with ClO_2 solutions or brine and then storage at 4 °C for 0, 3 or 7 days

Treatment	Day	Experiment #1			Experiment #2			Experiment #3		
		Replicate			Replicate			Replicate		
		I	II	III	I	II	III	I	II	III
Control	0	A	A	A	B	B	B	A	A	B
	3	B	B	B	B	C	B	B	B	C
	7	B	C[1]	C	B	C	B	B	B	C
Brine	0	A	A	A	B	B	B	B	A	A
	3	B	B	B	B	B	B	B	B	B
	7	C	C	C	C	C	C	B	B	C
20 ppm	0	B	B	B	B	C	B	B	B	B
	3	B	B	C	C	C	B	B	C	B
	7	C	C	C	C	C	C	C	C	B
40 ppm	0	C	C	C	C	C	C	B	C	B
	3	C	C	C	C	C	C	C	C	C
	7	C	C	C	C	C	C	C	C	C
100 ppm	0	C	C	C	C	C	C	C	C	C
	3	C	C	C	C	C	C	C	C	C
	7	C	C	C	C	C	C	C	C	C
200 ppm	0	C	C	C	C	C	C	C	C	C
	3	C	C	C	C	C	C	C	C	C
	7	C	C	C	C	C	C	C	C	C

[1]C means sunstandard.
U.S. substandard: whole fish does not possess reasonably good flavor and odor

unnaturally light color and the occurrence of a chocolate color in the gills were the major quality defects for the treated fish. Nontreated red grouper and salmon and those treated with brine also demonstrated some deterioration following storage for 7 days at 4 °C. The eye color changed, discoloration occurred with the skin and gills, and a fishy odor developed.

It must be pointed out that individual variation of test fish prior to treatment with the test solutions affected the results of the sensory trials, especially since the number of whole fish or fish fillets used in each experiment was limited. Quality variation of seafood samples also occurred between the batches used for different trials on different test dates, thereby affecting the overall evaluation results.

Conclusions

These results demonstrate that ClO_2 effectively removes microorganisms in water used for washing and handling seafood. Chlorine dioxide at a concentration of less than 40 ppm can be used practically as a sanitizing agent to reduce microbial loads and resolve odor problems of seafood. In other food processing operations, ClO_2 has been proven to successfully prevent buildup of microorganisms in water, accumulation of slime on equipment, and the development of undesirable odors. Therefore, ClO_2 can be used effectively as the washing water and flume water for handling seafood. Because the treated ClO_2 solution contains no microbial load, it can also be recycled during seafood processing.

CHAPTER IV
EFFECT OF CHLORINE DIOXIDE TREATMENT ON LIPID OXIDATION AND FATTY ACID COMPOSITION IN SALMON AND RED GROUPER FILLETS

Introduction

As people are becoming more aware of the relationship between diet and health, consumption of seafood has increased. Fish provide long-chain omega-3 polyunsaturated fatty acids which have been demonstrated to reduce platelet aggregation (Freese et al., 1994) and cardiovascular risk (Harris, 1989). Seafood fatty acids contain higher amounts of 20 and 22 carbon chain lengths and more highly unsaturated fatty acids than plants and animals (Stansby, 1982).

Aqueous chlorine has been used in the food industry to clean products including seafood, containers and equipment. However, due to health concerns of trihalomethanes and other chlorination reaction products generated during interaction of organics with aqueous chlorine, efforts have been made to explore alternatives. Chlorine dioxide (ClO_2) is a good candidate since it has a bactericidal efficacy equivalent to seven times that of aqueous chlorine in poultry processing chiller water (Lillard, 1979). Furthermore, less potentially toxic reaction products are produced following treatment of organic matter with ClO_2. Although ClO_2 has been tested with seafood and vegetables to enhance freshness and extend the shelf-life of products, little is known about its reaction with organic matter and its effect on nutrient content including fatty acid composition.

Chlorine dioxide is a potent oxidizer and an effective chlorinating agent. Therefore, the double bonds of fatty acids may undergo oxidation and halogen addition in the presence of ClO_2. The objective of this study was to investigate the effect of ClO_2 solutions at 20, 40, 100, and 200 ppm total available chlorine dioxide (TACD) in brine (3.5% NaCl solution) on oxidation and fatty acid compositions of red grouper and salmon fillets.

Materials and Methods

Fresh fillets of Atlantic salmon (*Salmo salar*) and red grouper (*Epinephelus morio*) were purchased from a local seafood store in Gainesville, FL. Fillets from red grouper and salmon weighing 1.8 and 2.7 kg, respectively, were cut roughly into 4-cm sections from anterior to posterior. The sections were further cut into cubes (4 x 4 x 4 cm). Fish cubes were mixed and randomly sampled for treatment with aqueous ClO_2 solutions or brine. Duplicate samples were used for each treatment group. The experiment was repeated.

Chlorine-demand-free water (CDF water) was prepared following the method of Ghanbari et al. (1982) by passing distilled water through two successive Barnstead deionizing units and then a glass column containing Porapak® Q (Supelco). This water was used to prepare all reagents. Aqueous solutions of ClO_2 were freshly prepared for each experiment from Oxine® Concentrate (OC, Bio-Cide International Co, OK) which contained 2% ClO_2 and 98% inert ingredients. A stock solution containing 2,000 ppm TACD was prepared by first reacting OC for 5 min at room temperature with 85% phosphoric acid at a 20:1 (v/v) ratio in a brown flask sealed with a glass stopper. The solution was then diluted with 9 volumes of ice-cold CDF water. This stock solution was used to prepare various working solutions (20, 40, 100 and 200 ppm TACD) in 3.5% ice-cold brine. Both the stock

and working ClO_2 solutions were freshly prepared on the day of the experiment. Aqueous ClO_2 concentrations were determined by iodometric titration and then by the *N,N'*-diethyl-*p*-phenylenediamine (DPD) ferrous titration method (American Public Health Association, 1989).

Duplicate fish cubes (25 g each) were treated separately by stirring for 5 min with ice-cold ClO_2 solutions in 3.5% brine at 20, 40, 100 and 200 ppm in beakers at a ratio of 1:5 (w/v). After the extra liquid was drained, the modified distillation-colorimetric method of Tarladgis et al. (1960) incorporating of antioxidants (i.e., propyl gallate) and chelating agents (i.e., EDTA) (Rhee, 1978) was used for estimating thiobarbituric acid reactive substances (TBARS) molar value (μmol MA/kg fish) in fish. Ten-gram portions of fish samples were homogenized with 35 mL of distilled water in a blender at high speed for 2 min, and the fish slurry was transferred to a 250 mL beaker. The blender jar was rinsed with 70 mL distilled water and the rinsing water was collected in the same beaker. After the slurry weight was adjusted to 110 g by adding distilled water, the pH of the sample was adjusted to 1.5 with a 4 N HCl solution. The sample was then transferred to a 500 mL round flask containing 100 mg each of propyl gallate and EDTA and a few anti-bumping granules. The sample was flushed with nitrogen gas. After 5 drops of antifoam B were added into the flask, the sample was distilled immediately using a vertical distillation assembly. Aliquots of 50 mL distillates were collected in volumetric flasks.

For the development of color, 5 mL of the distillate were pipetted into a screw capped tube. The blank was 5 mL distilled water. To prepare a standard curve, aliquots of 0, 0.4, 0.8, 1.0, 1.2, 1.6 and 2.0 mL of 1×10^{-5} M tetraethoxypropane (TEP, Sigma, St. Louis, MO)

standard solution were pipetted into screw capped tubes and then combined with distilled water to a volume of 5 mL. After 5 mL of TBA reagent was added into each of the tubes, they were mixed thoroughly using a vortex and then heated for 45 min in a vigorously boiling water bath. The tubes were cooled in tap water and the absorbance of the samples was determined at 538 nm using a Beckman DU-40 spectrophotometer (Fullerton, CA). The TBA values of the samples were calculated from a TEP standard curve (absorbance vs μmoles TEP) according the following equation:

$$\text{TBA (μmole/ kg fish)} = \frac{\text{T x V1 x 1000}}{\text{V2 x W}}$$

where T = μmoles of malonaldehyde (TEP) equivalent to the absorbance of a sample as determined from the standard curve; V1 = volume (mL) of distillate collected (50 mL); V2 = volume (mL) of distillate aliquot withdrawn for analysis (5 mL); and W = weight of fish added to the still (10 g).

For the fatty acid composition, the lipid was extracted by the procedure of Folch et al. (1957) as modified by Christie (1982). One-gram portions of fish sample were homogenized for 1 min with 10 mL of methanol in a Waring blender, then 20 mL of chloroform for another 2 min. After the fish slurry was filtered using a Whatman No. 1 filter paper on a Buchner funnel, the solid residue and filter paper were homogenized for 3 min with 20 mL chloroform plus 10 mL methanol. After filtration using the same procedures, the filtrates were pooled in a separatory funnel and added with 15 mL of a 0.88% aqueous KCl solution. The mixture was shaken thoroughly and the two phases were allowed to separate. After the upper layer was removed and the volume measured, it was washed with

one quarter volume of water. This washing procedure was repeated two more times, and the bottom layer containing purified lipid was added to an appropriate amount of sodium sulfate. After hydrated sodium sulfate was removed by filtration through a filter paper and then washed with chloroform, the pooled organic solvent in the round bottom flask was removed using a rotary evaporator (Rotovapor, R-110, Brinkmann, Switzerland) in a water bath at 45 °C. Fatty acid methyl esters (FAME) were prepared in screw capped tubes using the method of Maxwell and Marmer (1983). Briefly, 20 mg of lipid were added to 1 mL of isooctane and 100 μL of 2N KOH in methanol. The tubes were vortexed for 2 min. After a 2 min centrifugation at low speed, the bottom layer was removed and discarded and 0.5 mL of saturated aqueous ammonium acetate were added to the tubes. After mixing using a vortex, the tubes were centrifuged again and the bottom layer removed and discarded. The samples were then washed with 0.5 mL water, mixed and centrifuged again. After the water was removed, a small amount of sodium sulfate was added to the tubes. Samples in the top layer were removed to clean tubes, flushed with a stream of nitrogen gas, refrigerated for 30 min and then centrifuged. Sample supernatants were stored at -20 °C until analysis. A Sigma 3B gas chromatograph (Perkin Elmer) with split injector (1:40 ratio) and flame ionization detector was equipped with a DB-Wax column (30 m x 0.25 mm i.d., 0.25 μm film) and operated using helium as a carrier gas at a linear flow velocity of 25 cm/sec. The oven temperature was held at 195 °C for 12 min then programmed at 2 °C/min to 241 °C. The injector and detector temperatures were set at 300 °C. Fatty acids were identified by comparing their retention times with standards (Nu-Check Prep, Elysian, MN) and a cod

liver oil reference (Ackman, 1991). Individual fatty acids were reported as area %. Each sample was injected twice for analysis.

Analysis of variance (ANOVA) was performed using the general linear models procedure of the Statistical Analysis System (SAS Institute, 1985). Duncan's multiple range test was used for pairwise comparisons at a significance level of P=0.05.

Results and Discussion

Of the various components that affect quality attributes of fish, lipids are the most important. Lipids may undergo hydrolysis and oxidation reactions during processing and storage that adversely affect flavor, color and texture. The formation of carbonyl components and changes in fatty acid composition can serve as criteria for evaluation of oxidative rancidity in fish. Treatment of salmon and red grouper fillets for 5 min with ClO_2 solutions caused a dose-related increase in TBARS values (Figure 11). Those treated with 100 and 200 ppm ClO_2 had significantly ($P < 0.05$) greater TBARS values than the two controls and the 20 ppm group. Red grouper treated with 40 ppm ClO_2 also had a significantly ($P < 0.05$) greater TBARS value than nontreated and brine-treated samples. Ke et al. (1977) studied the lipid oxidation in various parts of frozen mackerel. The TBARS values in the white and dark muscles from mackerel increased from 2.4 and 4.2 μmol/kg fish to 6.8 and 8.2 μmol/kg fish, respectively, after storage at -15 °C for two months. Skin samples (subcutaneous fat) of mackerel increased from 5.8 to 58.9 μmol/kg fish. Compared to these data, the TBARS molar values of red grouper and salmon following treatment with ClO_2 solutions were only moderately elevated even though the treatment caused a dose-

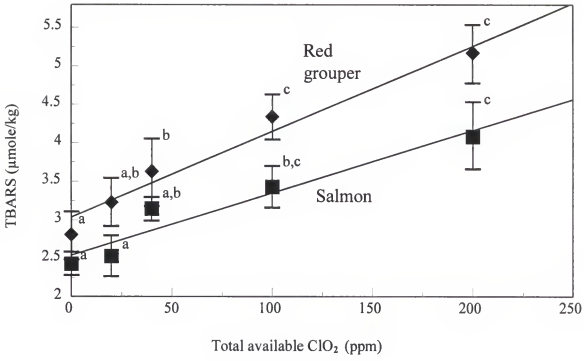


Figure 11. Effect of treatment with aqueous ClO_2 solutions on fish fillet TBARS values. Means ± standard deviation followed by different letters were significantly ($P < 0.05$) different from each other for each fish species. The brine-treated salmon and red grouper had respective TBARS values of 2.43 ± 0.62 and 2.81 ± 0.31 μmole malonaldehyde/kg fish.

related increase in TBARS values. Since we did not examine frozen storage of the treated fillets, it is unclear how this treatment would affect subsequent oxidation during storage.

Typical chromatograms of fatty acids from red grouper and salmon which were treated by brine are shown in Figures 12 and 13. Least square means of fatty acid composition following treatment with increasing levels of ClO_2 were compared (Tables 11 and 12). Lipids of salmon and red grouper fillets were highly unsaturated as expected. High levels of 20:5ω3, 22:5ω3 and 22:6ω3 were found in both species. Atlantic salmon had higher omega-3 polyunsaturated fatty acids (PUFAs) than red grouper. There were no obvious differences in fatty acid compositions that appeared to be a result of ClO_2 treatment (Tables 11 and 12). Highly unsaturated fatty acids (20:5ω3, 22:6ω3 and 20:4ω6) that would be expected to be sensitive to oxidizing conditions were not significantly ($P > 0.05$) affected by treatments ($P > 0.05$) in either fish species. Several of the fatty acids analyzed did appear to differ significantly due to treatment (in salmon, 17:0, 18:0, 18:2ω6, 18:3ω3, 18:4ω3, 20:4ω6, 20:4ω3 and 21:5ω3; in red grouper, 18:0, 20:2ω6, 22:4ω6 and 22:5ω3). However, in all of these cases, there was no clear effect of dose-response on fatty acid composition. The differences in fatty acid compositions between controls and treatments were minor. These results are probably attributable to experimental error in the fatty acid analysis and the unavoidable variations in fatty acid compositions among the cubed composite fish samples exposed to the various treatments. There appeared to be no effect of ClO_2 treatment on fatty acid compositions in salmon or red grouper under the experimental conditions examined.

TBARS were significantly ($P < 0.05$) but modestly elevated by ClO_2 treatment. The treated red grouper and salmon fillets did not differ in the percent monounsaturated and

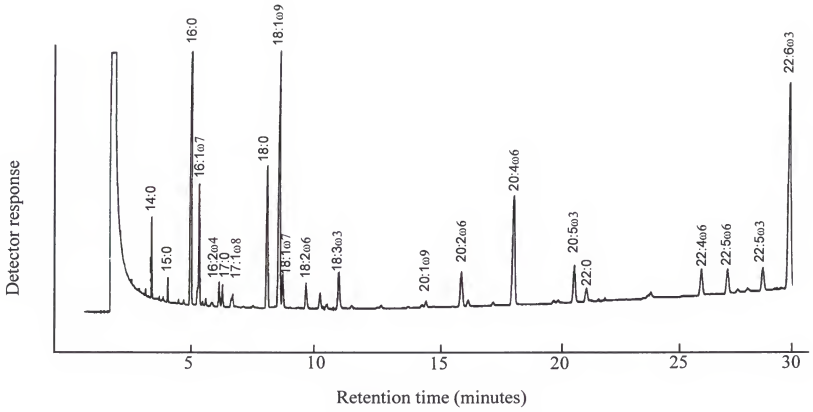


Figure 12. Typical chromatogram of fatty acid methyl esters from red grouper fillets treated with brine as control

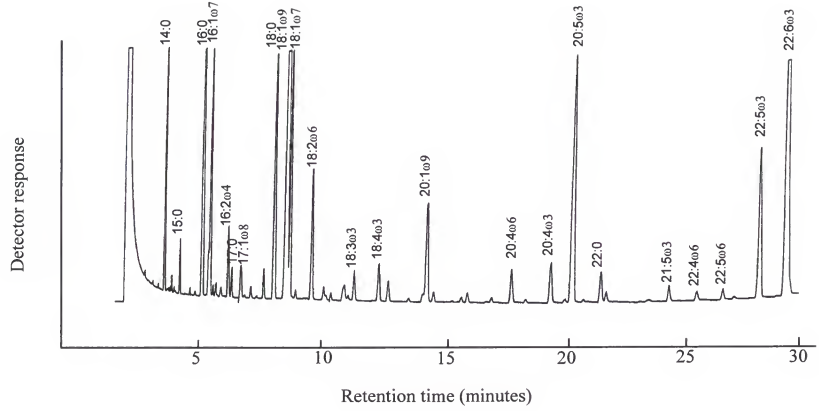


Figure 13. Typical chromatogram of fatty acid methyl esters from salmon fillets treated with brine as control

Table 11. Least square means of fatty acid compositions of red grouper fillets following treatment with aqueous chlorine dioxide

Fatty Acid	CT	BR	20	40	100	200	SEM	Effect of TRT (-P-)
14:0	2.12	2.21	1.82	1.98	1.70	1.64	0.206	0.2953
15:0	0.57	0.61	0.56	0.56	0.52	0.53	0.031	0.3748
16:0	22.2	22.8	23.6	22.9	21.9	22.0	0.664	0.4449
16:1ω7	5.76	5.19	4.80	4.84	4.58	4.15	0.397	0.1133
16:2ω4	0.97	0.95	0.95	1.04	0.95	0.94	0.042	0.5581
17:0	0.79	0.80	0.78	0.79	0.82	0.77	0.041	0.9648
17:1ω8	0.72	0.70	0.67	0.70	0.65	0.66	0.057	0.9434
18:0	6.41	6.70	6.62	6.90	6.48	6.73	0.101	0.0171
18:1ω9	14.6	14.1	14.1	14.4	13.1	13.1	0.473	0.1188
18:1ω7	2.23	2.03	1.95	2.14	1.90	1.78	0.114	0.0923
18:2ω6	1.22	1.32	1.31	1.25	1.21	1.33	0.054	0.4156
18:3ω3	0.56	1.28	1.41	1.11	1.22	1.31	0.198	0.0565
20:1ω9	0.70	0.86	0.65	0.76	0.58	0.61	0.076	0.1217
20:2ω6	0.74	1.78	1.45	1.25	1.23	1.09	0.131	0.0001
20:4ω6	6.89	6.67	7.28	7.51	7.75	7.93	0.379	0.1609
20:5ω3	2.80	2.56	2.43	2.56	2.97	2.52	0.129	0.0522
22:0	0.67	0.63	0.64	0.31	0.37	0.55	0.127	0.2365
22:4ω6	2.18	1.84	1.95	2.28	2.21	2.11	0.094	0.0178
22:5ω6	1.83	1.73	1.90	1.93	2.05	2.27	0.127	0.0737
22:5ω3	2.38	2.05	1.99	1.95	2.13	1.99	0.082	0.0068
22:6ω3	15.7	13.9	15.2	16.3	16.7	17.3	0.893	0.1058

CT: nontreated control; BR: brine-treated control; SEM: standard error mean;
Effect of TRT (-P-): effect of treatment on P value

Table 12. Least square means of fatty acid compositions of salmon fillets following treatment with aqueous chlorine dioxide

Fatty Acid	CT	BR	20	40	100	200	SEM	Effect of TRT (-P-)
14:0	4.06	3.79	4.29	3.75	3.88	4.12	0.151	0.1075
15:0	0.46	0.44	0.47	0.44	0.43	0.45	0.011	0.2214
16:0	16.0	16.2	15.1	16.6	15.5	15.6	0.353	0.0561
16:1ω7	6.31	5.67	6.71	5.89	5.94	6.23	0.241	0.0556
16:2ω4	1.04	1.00	1.06	1.03	1.01	1.03	0.024	0.4651
17:0	0.37	0.36	0.36	0.40	0.36	0.37	0.008	0.0241
17:1ω8	0.67	0.63	0.73	0.66	0.65	0.69	0.027	0.1690
18:0	4.16	4.11	4.04	4.25	4.09	4.14	0.058	0.0482
18:1ω9	20.2	19.2	21.3	19.6	20.3	20.6	0.544	0.1263
18:1ω7	3.71	3.80	3.58	3.99	3.78	3.74	0.100	0.1383
18:2ω6	2.39	2.28	2.52	2.27	2.36	2.45	0.061	0.0418
18:3ω3	0.62	0.61	0.64	0.59	0.62	0.63	0.009	0.0173
18:4ω3	0.84	0.82	0.92	0.77	0.83	0.86	0.026	0.0067
20:1ω9	2.65	2.52	2.74	2.57	2.66	2.66	0.082	0.5231
20:4ω6	0.86	0.89	0.81	0.86	0.87	0.84	0.018	0.0314
20:4ω3	1.12	1.10	1.16	1.05	1.12	1.13	0.023	0.0464
20:5ω3	7.33	7.47	7.20	7.01	7.25	7.20	0.140	0.3392
22:0	0.87	0.83	0.91	0.83	0.88	0.89	0.025	0.1719
21:5ω3	0.40	0.38	0.43	0.36	0.40	0.41	0.014	0.0255
22:4ω6	0.26	0.25	0.26	0.23	0.27	0.25	0.010	0.0985
22:5ω6	0.29	0.30	0.29	0.30	0.31	0.30	0.010	0.5875
22:5ω3	4.33	4.33	4.50	4.13	4.48	4.42	0.104	0.1599
22:6ω3	15.4	17.3	14.0	16.1	16.2	15.1	0.910	0.2024

CT: nontreated control; BR: brine-treated control; SEM: standard error mean; Effect of TRT (-P-): effect of treatment on P value

polyunsaturated fatty acids compared with nontreated controls, although differences in individual fatty acids occurred. These differences most likely were a result of experimental error as there is no easily understood way that ClO_2 would oxidize or add Cl to 18:1ω7 but not 18:1ω9. The differences in fatty acid composition within the fillets would likely also have contributed to the differences observed. According to Coppock et al. (1960), the treatment of flour with 280 ppm ClO_2 does not cause any immediate effect on fatty acid composition; the content of linolenic or arachidonic acid was not significantly ($P > 0.05$) changed. However, the use of too high a dose of ClO_2 may cause a color change in the flour by oxidizing the lipids (Moran et al., 1953; Meredith et al., 1956). Therefore, the results from our experiments indicate that ClO_2 treatment for 5 minutes did not affect the fatty acid composition of salmon and red grouper and resulted in very little lipid oxidation.

Conclusions

Chlorine dioxide-treated red grouper and salmon showed a dose-related increase in TBA; the 100 and 200 ppm groups had significantly ($P<0.05$) greater values for TBARS than controls and 20 ppm group. The values for TBARS of tested fillets after ClO_2 treatment for 5 min were only moderately elevated, even though the treatment caused a dose-related increase. Treated red grouper and salmon did not differ in percentage monounsaturated and polyunsaturated fatty acids compared to controls, although differences occurred with some individual fatty acids. Thus, ClO_2 treatment did not greater affect fatty acid composition of treated fillets.

CHAPTER V
EFFECT OF CHLORINE DIOXIDE TREATMENT ON THE NUTRITIONAL COMPOSITION OF SALMON AND RED GROUPER FILLETS AND ANALYSIS OF RESIDUAL CHLORINE DIOXIDE

Introduction

With the recent trend of increasing seafood consumption in the U.S., public health concerns have focused not only on ensuring product quality but also safety. Aqueous chlorine has been used in the seafood industry to sanitize seafood products. Due to safety concerns regarding formation of trihalomethanes and other chlorination reaction products following treatment with aqueous chlorine, chlorine dioxide (ClO_2) has been explored as a potential substitute for aqueous chlorine.

Chlorine dioxide is a strong oxidant; it is applied in a variety of processes including cellulose bleaching, and waste water and drinking water treatment. When ClO_2 reacts with aqueous contaminants, it is usually reduced to chlorite anion (ClO_2^-): $ClO_2 + e^- \rightarrow ClO_2^-$. Numerous reaction products from oxidation and chlorination can occur following treatment with ClO_2. For example, the reaction of ClO_2 with amines and alkenes could yield aldehydes as end products of chlorine oxidation (Stevens, 1982). Chlorine dioxide is a potent bactericidal agent. Its bactericidal effect in reducing bacterial populations in poultry chiller water and carcasses was tested (Tsai et al., 1995, Thiessen et al., 1984). Chlorine dioxide has a bactericidal efficacy equivalent to seven times that of aqueous chlorine in poultry

processing water (Lillard, 1979). Treatment with ClO_2 extended the shelf-life of broilers (Lillard, 1980) and reduced the incidence of *Salmonella* spp. on poultry carcasses (Thiessen et al., 1984). The bactericidal activity of ClO_2 is not affected by high pH or the presence of nitrogenous compounds (Benarde, 1965). Since less reaction products, including trihalomethanes, are produced in treated water, ClO_2 is believed to be a good substitute for aqueous chlorine in treating seafood, red meat, vegetables and fruits while maintaining their freshness and extending the shelf-life.

However, the use of ClO_2 in food processing is limited due to a lack of information related to its reaction with organic materials, the bactericidal efficiency in various food products, and its effects on nutrient content. Therefore, the objective of this study was to investigate the treatment of salmon and red grouper fillets with 20, 40, 100, and 200 ppm ClO_2 in brine (3.5% NaCl solution) on the contents of protein, lipid, calcium, phosphorous, iron, sodium, potassium, thiamine, riboflavin, and niacin. Furthermore, changes in moisture content and the uptake of chlorine species in treated samples were compared to those of nontreated and brine-treated controls. Such information is needed for regulatory approval of ClO_2 in treating seafoods while maintaining freshness and extending the shelf-life of these products.

Materials and Methods

Fish sample

Fresh Atlantic salmon (*Salmo salar*, high lipid, 15% fat content) and red grouper (*Epinephelus morio*, median lipid, 3% fat content) were purchased from a local seafood store during March and April. Whole fish were received on ice. Individual fish were filleted and

skinned so that no bones or skin were present, and the fillets were randomly sampled for treatment with aqueous ClO_2. The fillets were cut into cubes (about 2.5 x 2.5 x 2.5 cm). Fish cubes were mixed well and randomly sampled for treatment with aqueous ClO_2 solutions or brine. Duplicate samples were used for each treatment group. All treated cubes were stored at -30 °C and used within 3 days. Each cube was analyzed according to the flow scheme outlined in Figure 14 after its respective ClO_2 treatment was applied.

Chlorine-demand-free water

Chlorine-demand-free water (CDF water) was prepared following the method of Ghanbari et al. (1982) by passing distilled water through two successive Barnstead deionizing units (Dubuque, IA) and then a glass column containing Porapak® Q (Supelco, Bellefonte, PA). This water was used to prepare all reagents.

Preparation of stock chlorine dioxide and working solutions

Chlorine dioxide solutions were freshly prepared for each experiment from Oxine® concentrate (OC, Bio-Cide International, Norman, OK) which contains 2% chlorine dioxide and 98% inert ingredients. A stock solution containing 2,000 ppm available ClO_2 was prepared by reacting OC at room temperature with 85% phosphoric acid at a 20:1 (v/v) ratio for 5 min in a brown flask sealed with a glass stopper. The reaction mixture was then diluted with 9 volumes of ice-cold CDF water and titrated using the iodometric method. This stock solution was used to prepare various working solutions (20, 40, 100 and 200 ppm total available ClO_2 [TACD]) in ice-cold brine. Brine was prepared by dissolving 35 g NaCl in 1 liter of CDF water. Both the stock and working ClO_2 solutions were freshly prepared on the day of experiment.

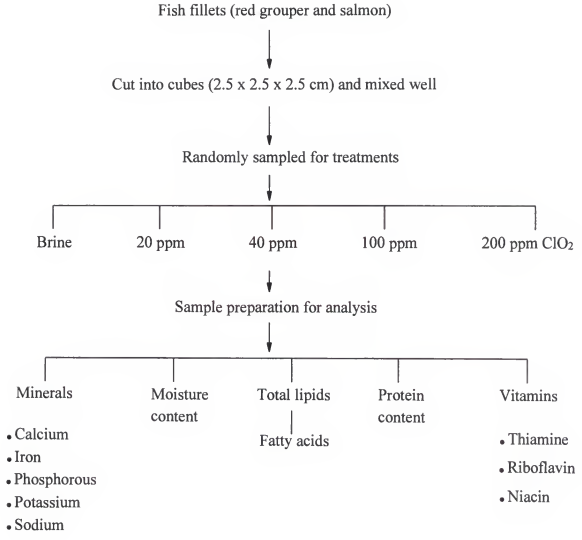


Figure 14. Flow scheme for nutrient analysis of chlorinated fish fillets

Titration of chlorine dioxide solutions with iodometric and *N,N'*-diethyl-*p*-phenylenediamine (DPD) methods

The iodometric method (APHA, 1989) was only used for determination of total available chlorine (TAC) in the test solutions. TAC is the sum of free and combined available chlorine (FAC and CAC, Jolley and Carpenter, 1983), while CAC is the chlorine present as NH_2Cl, $NHCl_2$, and organic N-chloro-compounds. FAC is the chlorine present as hypochlorous acid and hypochlorite ion. The DPD titration method using ferrous ammonium sulfate was employed to differentiate the individual chlorine species, including ClO_2, free available chlorine, monochloramine and dichloramine, and chlorite (Jolley and Carpenter, 1983; APHA, 1989). Since the optimal range of DPD titration was below 500 μg TAC, iodometric titration was conducted first to estimate TAC in test solutions. After the solutions were diluted to levels less than 500 μg TAC, DPD titration was conducted.

Iodometric titration. An iodometric titration method was used to determine TACD in stock and test solutions. In a glass-stoppered flask, an aliquot of test solution (1 mL for stock solution, or 1-2 mL for test solutions) was mixed with 15 mL of deionized distilled water, 5 mL of 25% potassium iodide (KI, Fisher Scientific), and 20 mL of 0.2 M HCl. After the mixture was kept in the dark for 2 min, it was titrated with sodium thiosulfate (0.1 N $Na_2S_2O_3$ for stock solution, and 0.01 N $Na_2S_2O_3$ for test solutions, Fisher Scientific) until the yellow color almost became clear. The mixture was placed in the dark for 5 min, after which a few drops (1 mL) of a 0.5% starch indicator solution was added. Titration with $Na_2S_2O_3$ was continued until the blue color disappeared. TAC and TACD were calculated as follows:

$$\text{TAC } (\mu g\ Cl_2/mL, ppm) = \frac{A \times N \times 35{,}450}{\text{volume of test sample (mL)}}$$

$$\text{TACD } (\mu g\ ClO_2/mL, ppm) = \frac{A \times N \times 13{,}490}{\text{volume of test sample (mL)}}$$

where A = volume of $Na_2S_2O_3$ used for titration, and N = normality of $Na_2S_2O_3$.

N,N-Diethyl-*p*-phenylenediamine (DPD) ferrous titrimetric method. The iodometric method can titrate only total available chlorine in the reaction mixture. Therefore, the DPD method was used to differentiate the individual chlorine species including free chlorine, chlorine dioxide, chlorite, monochloramine (RNHCl), dichloramine ($RNCl_2$), and nitrogen trichloride (NCl_3) (Jolley and Carpenter, 1983; APHA, 1989). The optimal range for DPD titration was from 0 to 500 µg TAC. Therefore, iodometric titration was applied first to estimate TAC in both stock and test solutions. Then, an aliquot containing < 500 µg TAC was used for DPD titration (APHA, 1989) to determine the content of each chlorine species.

DPD solution was freshly prepared on the day of testing by dissolving 1.1 g of *N,N*-diethyl-*p*-phenylenediamine sulfate salt (Sigma Chemical Co., St. Louis, MO) in 1 liter deionized distilled water containing 8 mL of 9 N H_2SO_4 and 200 mg of disodium ethylenediamine tetraacetate (EDTA disodium salt, Fisher Scientific). This solution was stored in a brown bottle.

To a 100 mL reaction mixture containing an aliquot of test sample (2-6 mL with < 500 µg TAC) and deionized distilled water (98-94 mL) was added 2 mL of a 10% glycine solution. This reaction mixture was then added to another reaction mixture containing 5 mL each of 0.5 M phosphate buffer (pH 6.2-6.5) and DPD, and 200 mg of EDTA. After mixing,

the solution was titrated rapidly with 100 ppm ferrous ammonium sulfate (FAS, LabChem Inc.) to a colorless endpoint (Reading G). The concentration of chlorine dioxide (μg/mL as Cl_2) in this sample was 5G.

To quantitate free available chlorine (FAC), another 100 mL reaction mixture was mixed with 5 mL each of 0.5 M phosphate buffer (pH 6.2-6.5) and DPD as well as 200 mg of EDTA. Titration with FAS was performed immediately until the red color disappeared (Reading A). To the mixture was added 1 g KI crystals and it was allowed to stand for 2 min before continuing the titration with FAS (Reading C).

Total available chlorine was determined by taking the reaction mixture from above (Reading C) and acidifying with 1 mL of a 5% H_2SO_4 solution. After standing for 2 min, 5 mL of a 5.5% sodium bicarbonate ($NaHCO_3$) solution was added. This was titrated to a colorless endpoint (Reading D). Chlorite was present in test samples if the D value was greater than C + 4G.

The concentration of the individual chlorine species (μg/mL as Cl_2) in test samples was calculated as follows: chlorine dioxide = 5G (or 1.9G when expressed as μg/mL ClO_2), free available chlorine = A - G, combined available chlorine = C - A, and total available chlorine = D. If chlorite was present, then the chlorite content was equal to [D - (C + 4G)]. Because 1 mL of 100 ppm FAS titrant used was equivalent to 100 μg chlorine, the free available chlorine, for example, contained in the stock solution could be calculated as:

$$\text{FAC} = \frac{(\text{A - G}) \times 100\ \mu\text{g FAS}}{\text{specified sample volume used (mL)}}$$

Determination of chlorine forms in treated solutions and fish cubes

Following treatment of fish fillets, the treated brine and ClO_2 solutions were titrated using DPD method to determine the composition of various chlorine forms. Excess liquid on the treated fish cubes was removed using a Whatman #1 filter paper and the samples were homogenized at high speed for 1 min in a blender with 4 volumes of CDF water (w/v). Following centrifugation at 1610 x g for 5 min in 50-mL centrifuge tubes, the supernatants were titrated by the iodometric and DPD methods. The pH of each test solution before and after treatment was also recorded. Furthermore, the pH of the supernatants was also checked.

Total protein contents

Duplicate fish portions (20 g each) were each treated with stirring for 5 min with 5 volumes of ice-cold ClO_2 working solutions (20, 40, 100, and 200 ppm) in beakers (1:5 w/v). Controls included nontreated fillets and those treated with brine. After draining excess liquid, the cubes were subjected to protein analysis using the modified macro-Kjeldahl method (AOAC, 1990). Total protein was calculated from Kjeldahl nitrogen using a 6.25 conversion factor. The experiments were repeated three times.

A 1.5-2.5 g sample from each fillet was weighed onto a weighing paper. The fish sample together with the weighing paper was then placed in a 500 mL Kjeldahl flask. A piece of weighing paper was used as a blank. After 10 g sulfate mixture (K_2SO_4 : Cu_2SO_4 = 10 : 1, w/w) and 30 mL of concentrated H_2SO_4 (36 N) were added to each flask, digestion with heating took place until the sample solutions turned to a light green color. After the samples were allowed to cool, four pieces of mossy zinc, several boiling chips and 250 mL of distilled water were added to each flask. The flask was then heated again for distillation.

Approximately 200-250 mL of distillate was collected and transferred into a 500 mL Erlenmeyer flask containing 100 mL of 4% boric acid solution and 12 drops of indicator (10 : 2 mixture of 0.1% bromocresol green and 0.1% methyl red in 95% ethanol). The collected samples were titrated with standardized 0.1 N HCl. Percent nitrogen and protein were calculated from the following equations:

$$\% N = \frac{(\text{mL HCl})\,(0.1 \text{ N HCl})\,(0.014)}{\text{g sample}} \times 100, \text{ and } \% \text{ protein} = (\% N) \times (6.25)$$

Total lipid contents

Total lipid was extracted from the homogenated mixtures of each treated cube (50 g) by the method of Bligh and Dyer (1959) and Folch et al. (1957). The experiments were repeated three times.

Fish samples (50 g ± 10 mg) were ground in a Waring blender (New Hartford, CT) with 50 mL of chloroform and 100 mL of methanol for 2 min. An additional 50 mL of chloroform was added to the blender and the sample was blended again for another 30 sec. Homogenization was repeated for another 30 sec after 50 mL of H_2O was added. The fish slurry was filtered into a 500 mL vacuum flask with a Buchner funnel using Whatman No. 1 filter paper. After the fish sample was pressed with a beaker to remove the solvent, fish residue and filter paper were blended again with 100 mL of chloroform for 30 sec. The sample was filtered as above into the same flask, and the blender was rinsed with 25 mL chloroform. The pooled filtrate was transferred to a 500 mL separatory funnel, and stood for 30 min to allow for complete separation of the two layers. The bottom layer was withdrawn into a 500 mL flat-bottom flask and the organic solvent was removed using a rotary

evaporator in a 45 °C water bath. After the lipid extract in the flask was flushed for 15 min with a stream of nitrogen gas to remove traces of chloroform, the lipid-containing flask was weighed. Total lipid content was calculated using the following equation:

$$\frac{\text{weight of flask with fat - weight of empty flask}}{\text{original sample weight}} \times 100 = \text{percent total lipids}$$

Moisture contents

The moisture content was determined using AOAC method 950.46 (1990). Fish samples of 2-3 g each were weighed into predried, preweighed aluminum moisture dishes (55 mm) and dried under vacuum for 18 hr at 95-105 °C. The moisture dishes were transferred to a desiccator for cooling, then weighed. The samples were dried for another 2 hr, cooled and weighed again. This process was repeated until the weights of the samples became constant. The total loss in weight was used to calculate the moisture content.

Vitamin analysis

Duplicate fish fillets (25 g each for thiamine and riboflavin analyses, 40 g each for niacin analysis) were each treated with ClO_2 and brine solutions as previously described. The experiments for vitamins were performed twice.

Thiamine. Thiamine was analyzed using the thiochrome method (AOAC 942.23, 1990; Ellefson, 1985). Ten-gram portions of ground fish samples were dispersed in 75 mL of 0.1 N HCl and autoclaved at 121 °C for 30 min. After the samples were cooled to 50 °C, 5 mL of 2 N sodium acetate was added to each sample to adjust the pH to 4.0-4.5. Following the addition of 5 mL of 2.5 M sodium acetate containing 10% α-amylase, the samples were incubated at 45-50 °C for 3 hr. After the samples were cooled to room temperature and the

pH adjusted to 3.5 using a 0.1 N HCl, the samples were brought to 100 mL with water and subjected to filtration through a Whatman No. 40 ash-free filter paper. Aliquots (25 mL) of the filtrates were passed through a previously washed Bio-Rex® 70 (50-100 mesh sodium form) resin column (Bio-Rad Laboratories, Hercules, CA). After the column was washed three times with 10 mL of almost boiling water, thiamine was eluted with five 4.0-4.5 mL aliquots of hot (60-70 °C) HCl-KCl solution into a 25 mL volumetric flask. The hot HCl-KCl solution was prepared by adding 8.5 mL concentrated HCl to 1 liter of 25% KCl solution. The 25 mL thiamine solution (0.2 μg/mL) serving as the standard was also passed through the Bio-Rex® 70 resin column.

Three aliquots of 5 mL each from the volumetric flask were pipetted into each of three 50 mL centrifuge tubes containing 1.5 g NaCl. After 3 mL of alkaline ferricyanide solution and 13 mL of isobutyl alcohol were added into two of the tubes (oxidized), they were shaken vigorously for 15 sec. To the third centrifuge tube (sample blank), 3 mL of 15% NaOH solution was added. Thiamine standard solution was processed similarly as the samples above. All centrifuge tubes, following the addition of isobutyl alcohol, were shaken again for 2 min and then centrifuged at low speed for 1 min. Five milliliters of the isobutyl alcohol extract (the upper layer) were decanted into cuvets and quantified for thiochrome, formed from the recovered thiamine, using a Sequoia-Turner Model 450 Digital Fluorometer (input filter: 360 nm; output filter: 440 nm; Abbott Diagnostics, Abbott Park, IL). The fluorescence of the isobutanol extracts of the oxidized (alkaline ferricyanide above) sample solutions was measured first (I), followed by the extracts of sample solutions which had been treated with 3 mL of 15% NaOH solution (sample blank, IB), then extracts of the oxidized

(alkaline ferricyanide above) standard thiamine solution (S), and finally, extracts of standard thiamine solution which had also been treated with 3 mL of 15% NaOH solution (standard blank, SB). The concentration of thiamine in fish sample was calculated from the following formula:

μg thiamine per g fish = (I - IB) / (S - SB) x 20 μg / fish weight

Riboflavin. The fish fillets were also subjected to riboflavin analysis using a fluorometric method (AOAC 970.65, 1990; Shah, 1985). Ten-gram portions of ground fish were dispersed in 75 mL of 0.1 N HCl and then autoclaved at 121 °C for 30 min. After the samples were cooled to room temperature and the pH adjusted to 6.0-6.5 using a 2 N NaOH solution, 1 N HCl was immediately added to bring the pH to 4.5. The samples were diluted to 100 mL with water and filtered through Whatman No. 40 ash-free filter papers. To a 50 mL aliquot of each filtrate was added dropwise 1 N HCl until no more precipitate was formed. After an equal number of drops of 1 N NaOH was added with constant shaking, the sample was diluted to 100 mL with distilled water. Ten milliliters of sample solution and 1 mL of water were added into each of 2 tubes and mixed. Another ten milliliters of sample solution and 1 mL of riboflavin working standard solution (0.5 μg/mL) were added into each of the other two tubes and mixed. One milliliter of glacial acetic acid was added into each of the 4 tubes and mixed. To each tube, 0.5 mL of 3% $KMnO_4$ was added and mixed, and the sample was allowed to stand for 2 min. After 0.5 mL of 3% H_2O_2 was added to each tube and mixed thoroughly, the samples were subjected to fluorescence measurement using a Turner Model 450 Digital Fluorometer (input filter: 440 nm; output filter: 535 nm). Deionized water was used as the blank. The fluorescence (A) of the extracts containing

added water was measured first. Then 20 mg of $Na_2S_2O_4$ was added with mixing to the tube and the fluorescence (C) of the solution was measured within 10 sec. Finally, the fluorescence (B) of the extracts containing added riboflavin was measured. Riboflavin content of the sample was calculated using the following formula:

μg riboflavin per g fish = (A-C)/(B-A) x 10/fish weight

Niacin. The fish fillets were subjected to niacin analysis using a colorimetric method (AOAC 961.14, 1990). A one-oz (28.35 g) portion of ground fish was dispersed in 200 mL of 1 N H_2SO_4 and autoclaved at 121 °C for 30 min. After the sample was cooled to room temperature and the pH adjusted to 4.5 using a 40% NaOH solution, it was diluted to 250 mL with distilled water and then filtered through a Whatman No. 40 filter paper. To a 50-mL volumetric flask containing 17 g ammonium sulfate, a 40 mL sample solution was added and then diluted to volume with distilled water. The solution was shaken vigorously, filtered again, and 1 mL of the filtrate was used for color development. The standard working niacin solution (4 μg/mL) was processed similarly.

One milliliter aliquots of niacin standard solution and sample solution were pipetted into respective tubes; to both the standard blank and sample blank were added 5 mL of water. All subsequent solutions were added to a single tube and the absorbance was read at 436 nm before proceeding with the next tube. Starting with the standard blank, the tube was mixed, then 0.5 mL of diluted NH_4OH (0.56-0.6% NH_3) was added and the tube was mixed again. Afterward, 2.0 mL of 10% sulfanilic acid was added and the tube was mixed; 0.5 mL of diluted HCl (1+5) was added, and the solution was mixed immediately and used for

calibration at 436 nm within 30 sec. A Beckman DU-40 spectrophotometer (Fullerton, CA) was used.

Standard solutions were treated in the same way as standard blanks with respect to the addition of 0.5 mL of diluted NH_4OH. The standard tube was mixed immediately, 5 mL of 10% CNBr solution was added and mixed. After 30 seconds, the tube was again mixed and 2.0 mL of 10% sulfanilic acid solution was added. After the tube was again mixed, 0.5 mL diluted HCl was added immediately and the tube mixed. With the absorbance of the instrument set at zero against the standard blank, the absorbance (As) of the standard solution was read at maximum. The absorbance (Au) of a sample solution was determined similarly, with the absorbance of the sample blank set at zero. Niacin content of the sample solution was calculated from the following formula:

mg niacin/100g fish sample = Au/As x R (μg)/1000 x dilution factor x 100 / weight of sample = Au/As x 3.2 (μg)/1000 x 250/40 x 50/1 x 100/28.3 = Au/As x 3.2 (μg) x 1.104; where Au = absorbance of sample solution, As = absorbance of standard solution, and R = weight of niacin taken in μg.

Mineral contents

Fish fillets (100 g) were treated for 5 min with stirring in a 3 L beaker with ice-cold ClO_2 solution in brine at 20, 40, 100, or 200 ppm at a ratio of 1:5 (w/v). Controls included nontreated fillets and those treated with brine. After the extra liquid was drained off, the fillets were placed in Whirl-Pak® bags (Nasco, Fort Atkinson, WI) and frozen at -20 °C. Samples were then sent on dry ice to the Food Laboratory, Florida Department of Agriculture and Consumer Services (Tallahassee, FL) for analysis.

Analyses for calcium, iron, phosphorus, potassium, and sodium were each performed using 5 g of treated red grouper and salmon fillets following the modified method of CEM Corporation's (Matthews, NC) Application Note FD-15 by the Food Laboratory. The sample was digested in an ultrapure nitric acid (16 N, 13 mL) and sulfuric acid (36 N, 2 mL) solution in a teflon digestion vessel using a microwave (630 W) as described in the application note. The digested test samples and mineral standards were subjected to analysis using a Perkin Elmer Plasma II Inductively Coupled Plasma instrument attached to an atomic emission spectrophotometer (ICP, Norwalk, CT). Triplicate fillets were used for each treatment condition and the experiment was repeated once.

Statistical analysis

Statistical analyses of the data were performed using analysis of variance (ANOVA) procedure with the Statistical Analysis System (SAS Institute, 1989). Duncan's multiple range test was used to determine if there were any significant differences between the means of the paired data at $P = 0.05$ level.

Results and Discussion

The pH of the test solutions decreased as the concentration of ClO_2 increased (Table 13). The interaction with fish fillets at a ratio of 5:1 (v/w) for 5 min caused significant ($P<0.05$) increases in pH of the brine and ClO_2 solutions (Table 13). The 200 ppm ClO_2 solution had a significantly ($P < 0.05$) lower pH value than any of the other treated solutions. Similar findings were reported by Lin et al. (1996) using ClO_2 solutions in Butterfield's buffer to treat mangrove snapper.

Table 13. Changes in pH of ClO_2 solutions following 5-min treatment of fish fillets

ClO_2 solution	Before treatment	After treatment with	
		red grouper	salmon
Brine	5.46 ± 0.35 (12)[1,B,a]	6.28 ± 0.16 (6)[a]	6.36 ± 0.10 (6)[A,a]
20 ppm	4.03 ± 0.40 (6)[B,b]	5.96 ± 0.10 (6)[b]	5.78 ± 0.30 (6)[A,b]
40 ppm	3.72 ± 0.26 (5)[C,b]	5.85 ± 0.16 (6)[b]	5.55 ± 0.12 (6)[B,b]
100 ppm	3.08 ± 0.26 (13)[C,c]	5.64 ± 0.17 (6)[c]	4.88 ± 0.20 (6)[B,c]
200 ppm	2.61 ± 0.31 (8)[C,d]	4.97 ± 0.10 (6)[d]	4.56 ± 0.21 (6)[B,d]

[1] Mean ± standard deviation. Number in parentheses indicates the number of samples tested.

[a,b,c] Within each column, means followed by different letters are significantly different at P=0.05.

[A,B,C] Within each row, means followed by different letters are significantly different at P=0.05.

The 40, 100 and 200 ppm ClO_2 solutions following treatment with salmon also had significantly ($P<0.05$) lower pH values than those treated with red grouper (Table 13). Differences in meat composition between the two fish species apparently affected the pH of the treated solutions.

The surface pH of red grouper fillets decreased significantly ($P < 0.05$) following treatment with brine or ClO_2 solutions (Table 14). For salmon fillets, the reduction of surface pH was significant ($P<0.05$) following treatment with 100 and 200 ppm ClO_2 solutions (Table 14). Again, differences in muscle composition probably contributed to variations in these pH values.

In most cases, the supernatants from fish samples treated with brine and ClO_2 solutions, which were prepared by homogenization and centrifugation of treated samples in CDF water, did not differ from each other (Table 14). These fish supernatants contained no available chlorine as determined by iodometric and DPD titration methods (data not shown).

About 66% of the total available chlorine (TAC) in a freshly prepared 200 ppm ClO_2 solution existed as chlorite and 29.5% as ClO_2 as shown by DPD titration (Table 15). Therefore, the actual content of ClO_2 in this solution was only 51.0 ppm (455 ppm x 29.5% ÷ 2.63). Only about 1% and 3.5% of the TAC existed as FAC and CAC, respectively. However, only ClO_2 was present in the 100 ppm ClO_2 solution. All the chlorite in this diluted and activated OC had been converted into 81.4 ppm ClO_2 (214 ppm x 100% ÷ 2.63). This discrepancy in the conversion rate of chlorite to ClO_2 in these two activated OC solutions was affected by chlorite content, pH, and reaction time. For example, all chlorite

Table 14. The pH of fish surfaces and fish supernatants following treatment with five volumes of ClO_2 solutions at various concentrations for 5 min

	Red grouper		Salmon	
ClO_2 solution	surface	supernatant	surface	supernatant
Fresh fish	6.63 ± 0.05 (31)[1,a]	ND	6.47 ± 0.11(41)^{a}	ND
Brine	6.35 ± 0.12 (7)^{b}	6.42 ± 0.08 (6)^{a}	6.45 ± 0.10 (7)^{a}	6.41 ± 0.09 (6)^{bc}
20 ppm	6.25 ± 0.10 (7)^{c}	6.32 ± 0.10 (6)^{ab}	6.36 ± 0.08 (7)^{ab}	6.34 ± 0.05 (6)^{c}
40 ppm	6.14 ± 0.07 (6)^{d}	6.31 ± 0.08 (6)^{ab}	6.36 ± 0.05 (6)^{ab}	6.31 ± 0.04 (6)^{c}
100 ppm	6.09 ± 0.17 (6)^{d}	6.29 ± 0.14 (6)^{b}	6.25 ± 0.13 (6)^{bc}	6.56 ± 0.12 (6)^{a}
200 ppm	5.83 ± 0.06 (6)^{e}	6.23 ± 0.05 (6)^{b}	6.18 ± 0.13 (6)^{c}	6.51 ± 0.15 (6)^{ab}

[1] Mean ± standard deviation. Number in parentheses indicates the number of samples tested.

ND: Not determined.

[a,b,c] Within each column, means followed by different letters are significantly different at P=0.05.

Table 15. Changes of residual molecular chlorine forms in ClO_2 solutions following treatment of salmon and red grouper for 5 min

Chlorine forms	Before treatment[a]		Treatment with salmon[b]				Treatment with red grouper[b]			
	100 ppm	200 ppm	20 ppm	40 ppm	100 ppm	200 ppm	20 ppm	40 ppm	100 ppm	200 ppm
ClO_2 as Cl_2 (%)	100	29.5 (3.07)	0	0	2.92 (3.52)	0	0	0	0.7 (1.71)	0
Chlorite (%)	0	66.0 (3.56)	98.0 (1.56)	98.7 (0.73)	93.4 (4.09)	98.5 (1.35)	96.0 (3.83)	98.7 (0.55)	97.4 (1.98)	99.9 (0.25)
FAC (%)	0	0.96 (0.13)	0.32 (0.87)	0.30 (0.56)	0.08 (0.24)	0	1.58 (2.24)	0.27 (0.42)	0.38 (0.43)	0
CAC (%)	0	3.51 (0.47)	1.64 (0.89)	0.99 (0.25)	1.96 (0.88)	1.19 (0.79)	2.47 (1.92)	1.17 (0.23)	1.57 (0.48)	0.10 (0.25)
TAC (ppm, DPD)	214 (2.45)	455 (40.7)	7.66 (4.28)	28.1 (5.73)	73.9 (2.73)	312 (29.0)	6.14 (4.08)	22.7 (4.98)	72.4 (6.64)	346 (5.89)
TAC (ppm, ID)	240 (26.6)	456 (51.8)	11.1 (3.27)	32.5 (5.41)	94.9 (4.15)	323 (40.0)	15.1 (4.62)	28.3 (3.48)	97.5 (5.21)	352 (13.5)

Abbreviations: FAC, free available chlorine; CAC, combined available chlorine; TAC, total available chlorine; DPD, *N,N*-diethyl-*p*-phenylenediamine titration; ID, iodometric titration.

[a] Mean (standard deviation) from four test samples, each consists of three readings.

[b] Mean (standard deviation) from six test samples, each consists of two readings.

in the 200 ppm ClO_2 solution in 3.5% NaCl (brine) was completely converted to ClO_2 following overnight storage at room temperature.

Treatment of fish fillets in these chlorinating solutions caused 24.0-85.7% reductions in TAC content (17.8, 33.1, 34.5 and 68.6% residual TAC in salmon-treated 20, 40, 100 and 200 ppm solutions respectively, and 14.3, 26.7, 33.8 and 76.0% for the respective red grouper treated solutions). Chlorite was the major molecular chlorine form present in treated solutions, accounting for 93.4-99.9% of total residual TAC, while FAC and CAC only accounted for 0.08-1.58% and 0.10-2.47%, respectively (Table 15). The presence of low levels of residual ClO_2 in the treated 100 ppm reaction solution was due to incomplete consumption of the original high content of ClO_2 in this solution for reaction with fish organic compounds (Table 15). Chlorite (ClO_2^-), FAC and CAC were formed following reaction of ClO_2 with fish organic compounds. No available chlorine was detected in brine solution before or after treatment with fish fillets (data not shown).

Control red grouper had a protein content of 19.6% (Table 16) which was similar to the reported value (Sidwell, 1981). Except for the salmon treated with 40 ppm ClO_2, treatment of salmon and red grouper fillets for 5 min with ClO_2 solutions at 20, 40, 100 and 200 ppm did not affect their protein contents. Salmon fillets treated with 40 ppm ClO_2 had a significantly lower protein content than controls and other treated groups (Table 16); this may be an artifact caused by error. No attempt was made to characterize the reaction of ClO_2 with fish proteins or their amino acid constituents. Proteins are subjected to oxidation, substitution, and addition reactions following treatment with aqueous ClO_2 (Jolley et al.,

Table 16. Effect of treatment with ClO_2 solutions at different concentrations on the total protein, lipid, moisture, and vitamins in salmon and red grouper fillets

Conc. (ppm)	Protein (%)	Lipid (%)	Moisture (%)	Thiamine (μg/10g fish)	Riboflavin (μg/10g fish)	Niacin (mg/100g fish)
Red Grouper						
Control	19.6 ± 0.95	2.61 ± 0.49[1d]	77.8 ± 1.52	16.3 ± 1.80[a]	25.6 ± 1.90[a]	2.36 ± 0.15[a]
Brine	19.6 ± 0.62	3.78 ± 1.33[ab]	78.5 ± 1.03	9.30 ± 2.40[b]	15.9 ± 2.50[bc]	1.81 ± 0.09[c]
20	19.3 ± 0.79	3.66 ± 0.93[ac]	78.5 ± 0.96	7.20 ± 0.50[c]	17.4 ± 2.30[b]	2.25 ± 0.15[ab]
40	19.6 ± 0.87	2.69 ± 0.86[bd]	78.6 ± 0.90	9.60 ± 0.50[b]	14.9 ± 2.20[bc]	2.25 ± 0.22[ab]
100	19.6 ± 0.48	3.16 ± 1.65[bcd]	78.6 ± 0.84	7.20 ± 0.40[c]	14.2 ± 0.70[c]	2.14 ± 0.16[b]
200	19.5 ± 0.80	2.48 ± 0.43[d]	78.4 ± 1.02	6.70 ± 0.70[c]	18.2 ± 3.30[b]	2.18 ± 0.22[ab]
Effect of TRT (-P-)	0.9681	0.1595	0.7879	0.0001	0.0001	0.0050
Salmon						
Control	21.0 ± 1.02[a]	15.7 ± 2.09[a]	72.0 ± 1.44[ac]	11.3 ± 0.55[a]	8.78 ± 1.51[ab]	4.76 ± 0.17[a]
Brine	20.7 ± 0.90[a]	12.7 ± 2.47[b]	74.3 ± 0.93[b]	9.08 ± 0.25[bc]	8.26 ± 0.91[ab]	4.14 ± 0.14[b]
20	21.4 ± 1.64[a]	14.6 ± 2.80[ab]	72.0 ± 1.23[ac]	8.76 ± 0.80[bc]	8.61 ± 0.46[b]	4.57 ± 0.36[a]
40	19.3 ± 0.23[b]	15.1 ± 2.50[ab]	71.9 ± 1.02[a]	9.37 ± 0.56[b]	7.28 ± 1.01[a]	4.49 ± 0.33[ab]
100	20.9 ± 1.09[a]	14.4 ± 2.16[ab]	73.4 ± 1.78[bc]	8.38 ± 0.47[c]	7.81 ± 0.38[a]	4.64 ± 0.07[a]
200	21.0 ± 1.51[a]	15.7 ± 0.93[a]	73.7 ± 0.78[b]	6.35 ± 0.32[d]	7.48 ± 0.92[a]	4.51 ± 0.24[a]
Effect of TRT (-P-)	0.0725	0.2279	0.0047	0.0001	0.2173	0.0359

[1] Within each column for each fish species, means ± standard deviation followed by different letters were significantly different from each other at P=0.05.

Effect of TRT (-P-): effect of treatment on P value

1978). Tan et al. (1987) showed that ClO_2 was inert towards individual amino acids except cysteine, tryptophan, and tyrosine at pH 3, and histidine and proline at pH 6.

Salmon fillets had a significantly ($P<0.05$) higher lipid content than red grouper (Table 16). Treatment of salmon fillets with brine caused a significant ($P<0.05$) reduction in total lipid content. Although salmon fillets treated with ClO_2, in general, had lower lipid contents than nontreated control, the difference was not significant ($P>0.05$). Red grouper fillets treated with brine and 20 ppm ClO_2 had significantly ($P<0.05$) higher lipid contents than the nontreated control and those treated with 200 ppm ClO_2. The reason for such variations in lipid content was unclear. Kim et al. (1997) showed that treatment of salmon and red grouper with aqueous ClO_2 caused dose-related increases in their thiobarbituric acid (TBA) values. However, no differences in fatty acid compositions were observed. The difference in lipid level probably reflected piece to piece or fish to fish variability. The salmon were cultured and had a higher fat content relative to the cold water salmon.

The moisture content of red grouper fillets was not affected by ClO_2 treatment (Table 16). The mean moisture content of untreated salmon and red grouper was 72% and 77.8%, respectively, which was close to the reported values of 74.5% and 78.6%, respectively (Sidwell, 1981). Salmon fillets treated with brine or 200 ppm ClO_2 had a significantly ($P<0.05$) higher moisture content than nontreated controls or those treated with 20 or 40 ppm ClO_2 (Table 16). Though salmon fillets treated with 100 ppm ClO_2 had a significantly ($P<0.05$) greater moisture content than those treated with 40 ppm ClO_2, they showed no difference compared to nontreated and brine-treated groups, and those treated with ClO_2 at 20 and 200 ppm.

Overall, the content of thiamine, riboflavin, and niacin in red grouper and salmon was reduced following treatment with brine or ClO_2 solutions because these vitamins are water soluble. The amount of riboflavin (256 μg/100 g) in control red grouper was lower than the reported value of 374 μg/100 g by Sidwell (1981). Conversely, the contents of niacin (2.36 mg/100g) and thiamine (163 μg/100 g) were higher than reported values of 1.40 mg/100 g and 374 μg/100 g, respectively. Thiamine (113 μg/100 g), riboflavin (87.8 μg/100 g), and niacin (4.76 mg/100 g) from the salmon control also differed from the reported values of 80 μg/100 g, 140 μg/100 g and 8 mg/100 g, respectively (Sidwell, 1981).

Treatment of salmon and red grouper fillets with brine and ClO_2 solutions caused a significant ($P<0.05$) reduction in thiamine content (Table 16). Except for the 40 ppm group, the reduction in thiamine content in ClO_2-treated red grouper and salmon were dose-related. Red grouper fillets treated with 200 ppm ClO_2 had the lowest thiamine content. Salmon fillets treated with 100 and 200 ppm ClO_2 had significantly ($P<0.05$) less thiamine content than the control and those treated with brine or 40 ppm ClO_2. Salmon fillets treated with brine, or 20 and 40 ppm ClO_2 showed no difference in thiamine content.

Control red grouper had a significantly ($P<0.05$) higher content of riboflavin than the treated samples with brine or ClO_2 (Table 16). Compared to the other treated groups, red grouper treated with 100 ppm ClO_2 had the lowest riboflavin content. Salmon fillets treated with ClO_2 solutions or brine also showed no significant ($P>0.05$) reduction in riboflavin content. The niacin content in red grouper and salmon fillets was significantly ($P<0.05$) reduced following treatment with brine. Except for the 100 ppm treated group, those red grouper samples treated with ClO_2 solutions at 20, 40, and 100 ppm did not differ from the

non-treated controls in niacin content. Salmon fillets treated with ClO_2 showed no difference in niacin content from the non-treated control. Good recoveries for thiamine, riboflavin, and niacin were obtained from salmon and red grouper fillets spiked with these vitamins (Table 17).

The calcium, iron, phosphorous, and sodium contents in control red grouper were 7.8, 2.4, 192.4, 404, and 45.3 mg/100g, respectively, which were similar to the reported values of 12.3, 0.3, 138.4, 444, and 46.7 mg/100g (Gall et al., 1983). The calcium, iron and phosphorus content of red grouper and the phosphorus and potassium content of salmon were not affected by treatment with brine or ClO_2 solution (Table 18). Treatment of red grouper with brine or ClO_2 solutions caused a decrease in potassium content. However, the differences between nontreated controls and ClO_2 treated samples were not significant ($P>0.05$). Treatment of red grouper and salmon with brine and ClO_2 solutions caused significant ($P<0.05$) increases in sodium contents. In most cases, the sodium content in the treated groups did not differ significantly ($P>0.05$) from each other. Such results were expected since ClO_2 solutions were prepared in 3.5% NaCl brine. Salmon fillets treated with 200 ppm ClO_2 had a significantly ($P<0.05$) higher calcium content than those treated with 20 ppm ClO_2. The reason for this increase in calcium content was not clear.

Conclusions

The treatment of salmon and red grouper with ClO_2 solutions in brine did not cause dramatic changes in their protein, fat, moisture, niacin, calcium, iron, phosphorus, and potassium content when compared to nontreated controls. Such treatment, however, caused a slightly reduction in the contents of thiamine and riboflavin, possibly because these two

Table 17. Recovery of thiamine, riboflavin, and niacin standards added to salmon and red grouper fillets

Thiamine added (µg/100 g fish)	% Mean ± SD (n=6)	Riboflavin added (µg/100 g fish)	% Mean ± SD (n=6)	Niacin added (mg/100g fish)	% Mean ± SD (n=6)
Red grouper					
10	83.0 ± 13.1	25	84.4 ± 6.7	1.06	99.2 ± 13.7
40	88.4 ± 13.1	50	83.5 ± 8.1	2.12	92.2 ± 1.7
80	97.5 ± 15.2	100	81.0 ± 5.5		
Salmon					
80	100.3 ± 11.5	50	86.3 ± 12.9	2.12	102.6 ± 15.1
100	90.8 ± 6.4	100	87.0 ± 7.3	4.24	86.1 ± 1.3
120	97.1 ± 12.2	200	84.2 ± 5.7		

Table 18. Effect of treatment with various concentrations of ClO_2 solutions on the mineral composition (ppm), wet weight basis, of salmon and red grouper fillets

ClO_2 solution	Calcium	Iron	Phosphorous	Potassium	Sodium
Red grouper					
No Treatment	78.0 ± 12.8[1]	2.39 ± 0.41	1924 ± 201.5	4040 ± 368.2^{a}	453.0 ± 101.1^{c}
Brine	72.3 ± 15.0	2.79 ± 1.90	1556 ± 163.0	3174 ± 315.6^{b}	1259 ± 113.0^{bc}
20 ppm	77.7 ± 17.7	2.35 ± 0.18	1744 ± 335.2	3395 ± 459.4^{ab}	2047 ± 867.0^{ab}
40 ppm	67.0 ± 5.57	1.60 ± 0.25	1721 ± 163.0	3462 ± 211.1^{ab}	2380 ± 572.2^{a}
100 ppm	61.7 ± 7.37	2.88 ± 0.29	1827 ± 324.6	3504 ± 650.5^{ab}	1535 ± 631.3^{ab}
200 ppm	61.0 ± 2.65	2.18 ± 0.30	1681 ± 105.8	3288 ± 266.2^{ab}	2020 ± 138.0^{ab}
Effect of TRT (-P-)	0.3263	0.4885	0.5229	0.2203	0.0063
Salmon					
No Treatment	76.0 ± 20.0^{ab}	1.64 ± 0.14^{ab}	2207 ± 173.0	3332 ± 277.0	551.3 ± 166.6^{b}
Brine	75.0 ± 7.00^{ab}	1.83 ± 0.42^{ab}	2129 ± 123.3	3196 ± 246.4	1773 ± 450.1^{a}
20 ppm	70.0 ± 10.4^{b}	1.76 ± 0.08^{ab}	2258 ± 160.8	3404 ± 284.9	1372 ± 377.4^{a}
40 ppm	83.7 ± 23.1^{ab}	1.56 ± 0.16^{b}	2097 ± 188.7	3147 ± 303.4	1321 ± 275.1^{a}
100 ppm	87.0 ± 15.6^{ab}	2.55 ± 0.93^{a}	2129 ± 384.5	3195 ± 697.4	1369 ± 403.1^{a}
200 ppm	102 ± 10.1^{a}	2.24 ± 0.64^{ab}	1975 ± 207.0	3339 ± 324.0	1811 ± 154.2^{a}
Effect of TRT (-P-)	0.2305	0.1954	0.7200	0.9514	0.0058

[1] Means ± standard deviation followed by different letters were significantly different from each other at P=0.05.

Effect of TRT (-P-): effect of treatment on P value

vitamins are water soluble and are susceptible to destruction by 3.5% NaCl. Since this is one of the first studies to determine the effect of ClO_2 treatment on the nutrition and proximate profiles of fish samples, the data obtained from this research will be useful for regulatory agencies, such as the FDA, in their evaluation process to approve ClO_2 as a substitute for aqueous chlorine for seafood treatment.

CHAPTER VI
DETERMINATION OF THE QUALITY, BACTERIAL LOADS, AND INORGANIC ANIONS IN TREATED SEAFOOD USING PRACTICAL LEVELS OF CHLORINE DIOXIDE

Introduction

The Food and Drug Administration (FDA) amended on March 3, 1995, the food additive regulations (21 CFR §173.69) to allow a 3 ppm residual chlorine dioxide (ClO_2) for controlling microbial populations in poultry processing water (FDA, 1995). Lillard (1979, 1980) reported that ClO_2 was an effective bactericide against *Salmonellae* in poultry chilling water and on broiler carcasses in one commercial processing plant in the United States. However, information is still limited regarding the usefulness of applying ClO_2 in the seafood industry to control microbial contamination problems.

Problems associated with the use of chlorine for water disinfection have occurred because of the formation of trihalomethanes and other potentially mutagenic/carcinogenic reaction byproducts. Chlorine dioxide could be used as a substitute for chlorine. It generally reacts as an electron acceptor. The H atoms in the organic C-H or N-H structures are not substituted by Cl following treatment with ClO_2. Additionally, in contrast to aqueous chlorine ($HOCl/OCl^-$), the efficiency of ClO_2 disinfection does not vary with pH or in the presence of ammonia. Chlorine dioxide is becoming more widely used in the food industry and is garnering regulatory approval for direct food contact. The growing use of this

alternative disinfectant has increased the need to monitor the levels of some potentially toxic byproducts, including chlorite (ClO_2^-) and chlorate (ClO_3^-).

The oxidation-reduction reactions of ClO_2 in water result in the formation of chlorite ion: $ClO_2 + e^- = ClO_2^-$. Chlorite ion is an effective oxidizing agent; it is consumed in oxidation-reduction reactions although at a much slower rate than ClO_2 under drinking water treatment conditions. This reduction is given by: $ClO_2^- + 4H^+ + 4e^- = Cl^- + 2H_2O$. During water treatment, approximately 50-70 percent of the ClO_2 used will immediately appear as ClO_2^- and the remainder as chlorine. The residual chlorite continues to degrade in the water distribution system via the above reaction, presumably in reaction with oxidizable material in the finished water or in the distribution system. The potential toxicity of ClO_2 metabolites (ClO_2^- and ClO_3^- ions) to the hemopoietic system is of interest from a human health perspective. Chlorite is recognized as the active species capable of causing hemolytic oxidative stress in animals (Couri et al., 1982). The U.S. EPA has recommended the use of chemically suppressed ion chromatography for the determination of chlorite, chlorate and other common inorganic anions. Most of the procedures were developed for the analysis of drinking water and have not been tested with rinse water following seafood treatment.

Since the results from previous studies indicated that the use of 100 or 200 ppm ClO_2 was deleterious to the sensory attributes of treated seafood, lower levels of ClO_2 at $\leq$ 30 ppm, which are more likely to be used in the seafood industry, were studied for potential seafood treatment. The objectives of this study were to (1) study the bactericidal effect of acidified sodium chlorite (activated ClO_2) in water used to wash seafood, (2) quantify residual chlorite (ClO_2^-) and chlorate (ClO_3^-) concentrations in the treated seafood products, and (3) determine

the sensory quality (appearance, texture, and odor) of the treated seafoods immediately following treatment and through refrigerated storage at 2 °C for 6 days. The seafood (sea scallops, shrimp, and mahi-mahi) was exposed for 1, 10, 60 and 180 min to tap water (24 °C) containing variable concentrations (0, 10, 20 and 30 ppm) of ClO_2.

Materials and Methods

Sample preparation

Three different types of seafood [sea scallops (*Placopecten magellanicus*), white penaeid shrimp (*Penaeus setiferus*), and mahi-mahi (*Coryphaena hippurus*)] were selected for this study in order to account for different products commonly exposed to water post during good manufacturing practices. All products were obtained fresh within 48 hours commercial harvest. Harvest included routine gear and on-board handling procedures employed for the respective fisheries.

Sea scallops were selected to represent a common, popular molluscan shellfish. The products were harvested and initially packaged in Boston prior to frozen shipment to Gainesville, FL. The edible meats or adductor muscle (40-50 count/pound) had been previously hand-shucked, washed and packed in customary 5 lb wax boxes with polybag liners. These products are typically exposed to a series of washes to remove any visceral fragments and surface seepage.

White penaeid shrimp was selected to represent a common, popular crustacean shellfish. The product form was shell-on tails (count: 30-35 tails/pound). The shrimp had been harvested by trawler in the Atlantic ocean adjacent to St. Augustine, FL, then frozen in 5 lb boxes prior to shipment and storage in Gainesville, FL.

Mahi-mahi was selected to represent a common, popular marine fish. The product was harvested by longline vessels operating in the Pacific ocean off Quepas, Costa Rica. The gutted fish were shipped fresh to Gainesville, FL. The product form was whole, skin-on fish. The fish were screened for quality (no defects noted) then frozen (-20°C). The thawed fish were skinned and cut to fillet portions (approx. 400-450 g/fillet; approx. 3 cm thick). The lateral dark or red muscle tissue remained as an integral part of the fillets. These fillets were more representative of a pelagic fish with more obvious red muscle tissue. This tissue is more vulnerable to oxidation and related changes in quality, due to the amount, character and distribution of lipids. This selection assured observations for a more sensitive fish product.

Preparation of activated ClO_2 stock solution

The ClO_2 stock solution was activated by mixing 15 mL 85% phosphoric acid (Fisher Scientific, Certified ACS) for 5 min at room temperature with 300 mL Oxine® concentrate (Bio-Cide International, Inc., Norman, OK) in a brown flask sealed with a glass stopper. The reaction mixture was then diluted with 9 volumes of tap water. This stock solution was used to prepare various test solutions (10, 20, 30 ppm ClO_2) with tap water based on its content of total available ClO_2 (TACD expressed as μg/mL ClO_2) determined by iodometric titration method (APHA, 1989). The concentrations of total available chlorine (TAC), TACD, and various chlorine species in stock and test solutions were determined by iodometric and *N,N*-diethyl-*p*-phenylenediamine (DPD) ferrous titrimetric methods (APHA, 1989). The stock and test solutions used to treat sea scallops and mahi-mahi were freshly prepared on the day of testing. This same stock solution was stored overnight and then used to prepare solutions for treating shrimp.

Treatment of seafood with ClO_2 solutions

The various seafood samples, sea scallop meats, shrimp tails and mahi-mahi fillets, were exposed in coolers for 1, 10, 60 and 180 min to tap water (24 °C) containing 0, 10, 20 or 30 ppm ClO_2. The tap water quality in Gainesville, FL is as follows (Gainesville Regional Utilities, 1997): total hardness: 100-120 mg/L or 6 to 7 grains; free chlorine residual in system: 0.5 mg/L to 0.8 mg/L; fluoride residual: 0.75 mg/L; sodium content: 10 mg/L to 15 mg/L (average 12 mg/L); and pH: 8.6 to 8.8. The treatments involved exposure of approximately 5 kg of product immersed directly into 10 liters of the respective ClO_2 concentrations. At each time interval, subsamples (approximately 1 kg) of treated seafood were removed. One third of the subsamples were used to determine bacterial load and to quantify chlorite and chlorate. The remaining two thirds, used for sensory evaluation, were divided equally into duplicate-covered trays for refrigeration storage at 2 °C. Untreated seafood samples were used as controls.

Concurrently, samples of all solutions (100 mL) were obtained in 50 mL centrifuge tubes in sequence (0, 1, 10, 60 and 180 min) following removal of seafood samples for the determination of bacterial loads. Aliquots of all solutions (100 mL) before seafood treatment were used to determine ClO_2 content using iodometric and DPD titration methods.

Sensory evaluation of treated seafood

Sensory assessment of the seafood samples prior to and following ClO_2 treatment were preformed by a 3 member panel of experts. Their expertise was based on over 35 years of combined experience in commercial and analytical settings assessing seafood quality. Their experience is necessary in their daily research and extension services through the Food

Science and Human Nutrition Department, University of Florida. Their descriptive ratings were based on direct product observation and comparison among treatments prior to exposure and subsequent to exposure, immediately and after 1, 2, 5, and 6 days of storage at 2 °C. The descriptions were based on direct comparisons with the untreated (0 min exposure) or control products.

Determination of bacterial loads in treated solutions and seafood samples

Treated solutions collected at each time interval with each test concentration were serially diluted ten-fold with Butterfield's buffer. Aliquots (0.1 mL) from each diluted sample were surface plated on quadruplicate tryptic soy agar (TSA, Difco Laboratories, Detroit, MI) plates containing 1.5% NaCl. In addition, pour plate method was also used for the test solutions. The plates were incubated at room temperature for 3 days before colonies were counted.

For treated seafood, a 25-g portion of the samples collected at each time interval with each test concentration was homogenized at high speed for 2 min in a sterile blender jar with 100 mL (for sea scallops) or 225 mL (for shrimp and mahi-mahi) of sterile Butterfield's buffer. The homogenate was then serially diluted ten-fold in Butterfield's buffer. Aliquots (0.1 mL) from each diluted sample were surface plated on quadruplicate TSA plates containing 1.5% NaCl. Pour plate method was also used with the homogenates. Bacterial colonies on these plates were counted after incubation at room temperature for 3 days. The untreated seafood controls were processed similarly to determine initial bacterial loads.

Quantitation of chlorite and chlorate in treated seafood samples

The seafood homogenates in Butterfield's buffer were transferred to 50 mL centrifuge tubes and centrifuged at 15,000 x g for 40 min using an IEC B-20A Centrifuge (Damon/IEC

Division, Needham Heights, Mass) at 4 °C. The supernatants were removed and filtered through 0.2 µm Acrodisc filters (Gelman Sciences, Ann Arbor, MI). These filtrates were then stored at 4 °C until analysis for chlorite and chlorate using a Dionex 2010i ion chromatograph (Sunnyvale, CA). The USEPA Method 300 "Determination of Inorganic Anions in Water by Ion Chromatography" was followed. The columns used were an IonPac AS9-SC analytical column (4 mm, Dionex, Sunnyvale, CA) and IonPac AG9-SC guard column (4 mm). The eluent was 1.4 mM Na_2CO_3/0.2 mM $NaHCO_3$; the flow rate was 1.5 mL/min; and the injection volume was 100 µL. An Anion Self-Regenerating Suppressor was connected to the system and the detection was suppressed conductivity in an autosuppression recycle mode.

The mixtures of sodium chlorite and sodium chlorate standard solutions were prepared in distilled and purified water (Photronix Water System [Reagent Grade]: Photronix Corp., Medway, MA). Concentrations of each ion at 0.125, 0.25, 0.5, 0.75, 1.0 and 1.5 ppm were run daily. Curves showed linear responses (R^2 = 0.999) for the dose-peak height relationship. Each test sample was injected twice with the averaged values reported. All glassware used in this experiment was washed with purified water.

Results and Discussion

Contents of various chlorine species in stock and test solutions

Freshly prepared stock solutions of acidified sodium chlorite had 45.5-63.9% ClO_2, 0.02-0.035% FAC, 19.5-20.3% CAC, and 14.3-30.1% chlorite (Tables 19 and 20). However, the 1-day old stock solution had 89.8% ClO_2, 0.016% FAC, 20.3% CAC, and no chlorite (Table 21). Most of the chlorite in the stock solution used to prepare test solutions for the shrimp treatment was converted to ClO_2 after overnight storage.

Table 19. Various chlorine species present in test and stock solutions used for the treatment of sea scallops as determined by iodometric and DPD titration methods

	Tap water	Test solutions			Stock solution
		10 ppm	20 ppm	30 ppm	
Iodometric	ppm				
TAC as Cl_2	0	35.5	49	70.9	4112
TACD as ClO_2	0	13.5	18.9	27	1564
DPD	ppm				
Total ClO_2 as Cl_2	0	10 (39.1)	28.8 (55.4)	41.7 (54.9)	1300 (45.5)
Total ClO_2	0	**3.8**	**11.0**	**15.9**	494
FAC	0	0.2 (0.78)	0.3 (0.58)	0.3 (0.39)	120 (4.20)
CAC	0	6.8 (26.6)	13 (25.0)	19.7 (25.9)	580 (20.3)
Chlorite	0	8.6 (33.6)	10 (19.2)	14.3 (18.8)	860 (30.1)
TAC	0	25.6 (100%)	52 (100%)	76 (100%)	2860 (100%)
TAC as ClO_2	0	9.7	19.8	28.9	1087

The test solutions (10, 20, and 30 ppm ClO_2) were prepared by diluting the stock solution with tap water based on its TACD (1564 ppm ClO_2) as determined by iodometric method. Each of these test solutions was then titrated by iodometric and DPD methods for TACD and individual chlorine species. The stock solution was also titrated by DPD method to quantify each chlorine species. The number in parenthesis is the percent content of each chlorine species in the ClO_2 solution.

Table 20. Various chlorine species present in test and stock solutions used for the treatment of mahi-mahi as determined by iodometric and DPD titration methods

	Tap water	Test solutions			Stock solution
		10 ppm	20 ppm	30 ppm	
Iodometric	ppm				
TAC as Cl_2	0	35.5	74.4	95.7	4431
TACD as ClO_2	0	13.5	28.3	36.4	1618
DPD	ppm				
Total ClO_2 as Cl_2	0	20 (64.9)	43.3 (65.1)	67.5 (73.8)	3400 (63.9)
Total ClO_2	0	**7.6**	**16.5**	**25.7**	1293
FAC	0	0 (0)	3.3 (4.96)	0.5 (0.55)	120 (2.26)
CAC	0	6.5 (21.1)	18.5 (27.8)	23.5 (25.7)	1040 (19.5)
Chlorite	0	4.3 (14.0)	1.3 (1.95)	0 (0)	760 (14.3)
TAC	0	30.8 (100%)	66.5 (100%)	91.5 (100%)	5320 (100%)
TAC as ClO_2	0	11.7	25.3	34.8	2023

The test solutions (10, 20, and 30 ppm ClO_2) were prepared by diluting the stock solution with tap water based on its TACD (1618 ppm ClO_2) as determined by iodometric method. Each of these test solutions was then titrated by iodometric and DPD methods for TACD and individual chlorine species. The stock solution was also titrated by DPD method to quantify each chlorine species. The number in parenthesis is the percent content of each chlorine species in the ClO_2 solution.

Table 21. Various chlorine species present in test and stock solutions used for the treatment of shrimp as determined by iodometric and DPD titration methods

	Tap water	Test solutions			Stock[1] solution
		10 ppm	20 ppm	30 ppm	
Iodometric	ppm				
TAC as Cl_2	0	47.3	70.9	85.1	3332
TACD as ClO_2	0	18	27	32.3	1267
DPD	ppm				
Total ClO_2 as Cl_2	0	33 (81.3)	61.3 (83.9)	91.7 (81.4)	2300 (80.4)
Total ClO_2	0	**12.5**	**23.3**	**34.9**	875
FAC	0	0.2 (0.49)	2 (2.74)	4.7 (4.17)	40 (1.40)
CAC	0	7.4 (18.2)	9.8 (13.4)	16.3 (14.5)	520 (18.2)
Chlorite	0.5	0 (0)	0 (0)	0 (0)	0 (0)
TAC	0.5	40.6 (100%)	73.1 (100%)	112.7 (100)	2860 (100)
TAC as ClO_2	0.2	15.4	27.8	42.9	1087

[1] This stock solution for shrimp treatment is the overnight storaged (at room temperature) stock solution for sea scallops treatment.

The test solutions (10, 20, and 30 ppm ClO_2) were prepared by diluting the stock solution with tap water based on its TACD (1267 ppm ClO_2) as determined by iodometric method. Each of these test solutions was then titrated by iodometric and DPD methods for TACD and individual chlorine species. The stock solution was also titrated by DPD method to quantify each chlorine species. The number in parenthesis is the percent content of each chlorine species in the ClO_2 solution.

The 10, 20, and 30 ppm ClO_2 solutions were prepared on each test date from stock solutions based on results of iodometric titration. However, such test solutions, when titrated by DPD method, were found to have respective values of 3.8, 11 and 15.9 ppm ClO_2 for treatment of sea scallops (Table 19); 7.6, 16.5 and 25.7 ppm ClO_2 for treatment of mahi-mahi (Table 20); and 12.5, 23.3 and 34.9 ppm ClO_2 for treatment of shrimp (Table 21). Therefore, the use of iodometric titration method to prepare testing ClO_2 solution is not appropriate for industrial practice.

Changes of bacterial loads in treated solutions with seafood

Compared to tap water, the treated test solutions showed dose- and time-related changes in bactericidal activity following immersion of sea scallops, mahi-mahi or shrimp (Figures 15-17). The seafood affected the bactericidal efficacy of the test solutions. Test solutions containing 11 and 15.9 ppm ClO_2 for sea scallops and 12.5 ppm ClO_2 for shrimp killed all bacteria in the suspensions following 1 min of treatment, but failed to kill the bacteria after the seafood was treated for 10 min (Figures 15 and 17). However, the test solution containing 16.5 ppm ClO_2 killed all bacteria in the solution after mahi-mahi was treated for 10-60 min (Figure 16). Apparently, the activated ClO_2 reacted more readily with sea scallops and shrimp than with mahi-mahi. The interaction of ClO_2 with seafood proteins or organics in the test system may have reduced the amount of available ClO_2 for bactericidal activity. These surviving bacteria then increased in numbers as time proceeded.

Water discoloration

The treated solutions showed noticeable changes in clarity and color during exposure to seafood. The control solution (0 ppm exposure) for sea scallops showed no change in

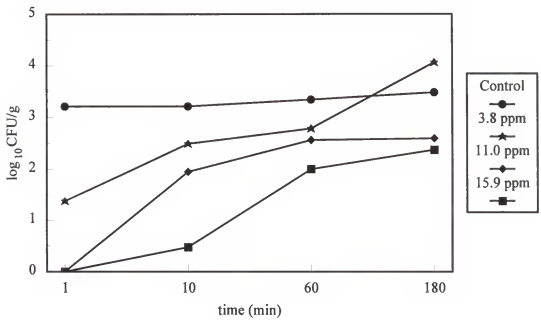


Figure 15. Time- and dose-related changes of bacterial loads in test ClO_2 solutions following treatment of sea scallops

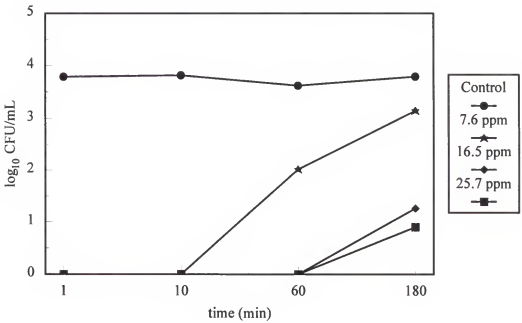


Figure 16. Time- and dose-related changes of bacterial loads in test ClO_2 solutions following treatment of mahi-mahi

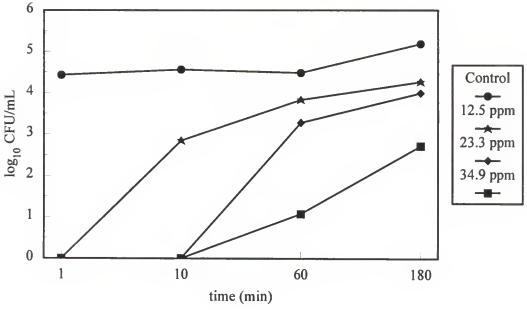


Figure 17. Time- and dose-related changes of bacterial loads in test ClO_2 solutions following treatment of shrimp

color. However, the treated ClO_2 solutions at 3.8, 11.0 and 15.9 ppm changed to pink, red and dark red, respectively, within 10 min of exposure. The control solution for shrimp was slightly cloudy after a 60 min exposure time. The 12.5 ppm ClO_2 solution appeared pinkish-brown after treatment of shrimp for 2 min. It then progressively changed to light brown after 180 min. This progressive change of color also occurred with the 23.3 and 34.9 ppm ClO_2 solutions, but the initial (1 min) and final (180 min) colors were more intense and the solutions were more opaque. The control and test solutions for mahi-mahi became cloudy in a similar manner to shrimp, and became more opaque with increasing exposure time and concentrations (7.6-25.7 ppm ClO_2).

Changes of bacterial loads on seafood treated with ClO_2

As with the tap water, the test solutions at 3.8-34.9 ppm ClO_2 did not cause dramatic reduction of bacterial loads on treated sea scallops, shrimp or mahi-mahi (data not shown). Most of the treated seafood samples showed no decrease in bacterial loads after treatment for 3 hours. This failure to effectively kill bacteria on seafood samples allowed bacterial growth to take place in test solutions during the 3-h period.

Contents of chlorite and chlorate in treated seafood

The separation of chlorite and chlorate in the seafood samples was accomplished using a 1.4 mM Na_2CO_3/0.2 mM Na_3HCO_2 eluent system commonly used to resolve anions such as chlorite and chlorate (Figure 18). Figure 19 shows the standard curves revealing the linear relationships of peak height (cm) versus the concentrations of chlorite and chlorate.

Tables 22-24 show the contents of chlorite and chlorate in sea scallops, mahi-mahi, and shrimp following treatment for various time periods with test solutions. No chlorite

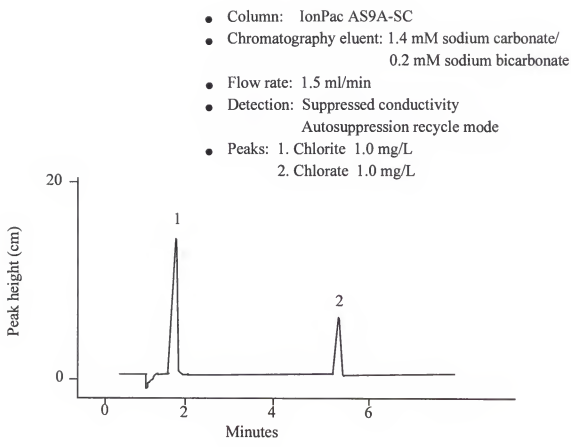


Figure 18. Separation of inorganic anions (chlorite and chlorate) on an IonPac AS9A column

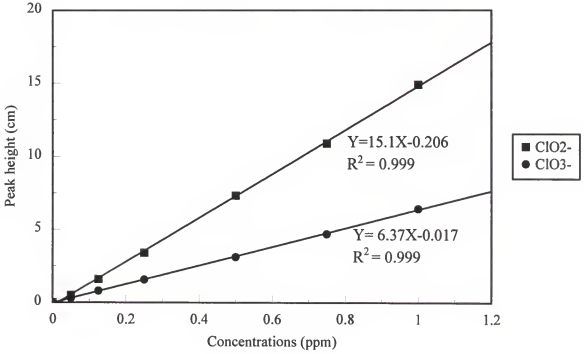


Figure 19. Standard curves of chlorite (ClO_2^-) and chlorate (ClO_3^-) by ion chromatography

Table 22. Time-related changes of chlorite (ClO_2^-) and chlorate (ClO_3^-) concentrations in sea scallops homogenates following treatment with 3.8, 11.0, and 15.9 ppm ClO_2 solutions

Solution	ClO_2^- and ClO_3^-	1 min	10 min	60 min	180 min
Water	ClO_2^- (μg/g)	0	0	0	0
	ClO_3^- (μg/g)	0	0	0	0
3.8 ppm	ClO_2^- (μg/g)	0	0	0	0
	ClO_3^- (μg/g)	0	0	0	0
11.0 ppm	ClO_2^- (μg/g)	0	0	0	0
	ClO_3^- (μg/g)	0	0	3.26	2.60
15.9 ppm	ClO_2^- (μg/g)	0	0	0	0
	ClO_3^- (μg/g)	0	2.98	6.35	9.30

No chlorite and chlorate was detected in the homogenate of non-treated scallops.

Table 23. Time-related changes of chlorite (ClO_2^-) and chlorate (ClO_3^-) concentrations in mahi-mahi homogenates following treatment with 7.6, 16.5, and 25.7 ppm ClO_2 solutions

Solution	ClO_2^- and ClO_3^-	1 min	10 min	60 min	180 min
Water	ClO_2^- (μg/g)	0	0	0	0
	ClO_3^- (μg/g)	0	0	0	0
7.6 ppm	ClO_2^- (μg/g)	0	0	0	0
	ClO_3^- (μg/g)	0	0	0	1.80
16.5 ppm	ClO_2^- (μg/g)	0	0	0	0
	ClO_3^- (μg/g)	1.00	3.30	3.20	6.14
25.7 ppm	ClO_2^- (μg/g)	0	0	0	0
	ClO_3^- (μg/g)	1.50	3.55	6.10	9.24

No chlorite and chlorate was detected in the homogenate of non-treated mahi-mahi.

Table 24. Time-related changes of chlorite (ClO_2^-) and chlorate (ClO_3^-) concentrations in shrimp homogenates following treatment with 12.5, 23.3, and 34.9 ppm ClO_2 solutions

Solution	ClO_2^- and ClO_3^-	1 min	10 min	60 min	180 min
Water	ClO_2^- (μg/g)	0	0	0	0
	ClO_3^- (μg/g)	0	0	0	0
12.5 ppm	ClO_2^- (μg/g)	0	0	0	0
	ClO_3^- (μg/g)	0	0	0	0
23.3 ppm	ClO_2^- (μg/g)	0	0	0	0
	ClO_3^- (μg/g)	1.14	1.71	2.54	2.59
34.9 ppm	ClO_2^- (μg/g)	0	0	0	0
	ClO_3^- (μg/g)	1.32	1.97	3.55	4.71

No chlorite and chlorate was detected in the homogenate of non-treated shrimp.

residue was found in any of the treated sea scallops, mahi-mahi, or shrimp. Low levels of chlorate residues were detected in some of the treated samples. In most cases, the increase in chlorate content in treated seafood at a defined ClO_2 concentration was time-related or dose-related at a defined treatment period. The minimal detection limit of chlorate in sea scallops, mahi-mahi, and shrimp was 1.0, 2.0 and 1.0 ppm (μg/g), respectively. The minimal detection limits of chlorite and chlorate in deionized water was 0.05 ppm.

However, this low level of ClO_3^- found in treated seafood products can also be changed to other chlorine species. The FDA evaluated the petitioned data and determined that very low levels of chlorite and chlorate, retained on fresh poultry carcasses as a result of exposure to processing water containing ClO_2 for controlling microbial populations, would be converted to correspondingly low levels of chloride during cooking (Food Chemical News, 1995). Therefore, the chlorate residue detected in treated seafood poses little or no health hazard to consumers.

Sensory evaluation of treated seafood

Sensory assessments of sea scallops indicated no discernible differences between products exposed to water or to activated ClO_2 until the exposure to ClO_2 at 3.8 - 15.9 ppm exceeded 10 min (Table 25). The noted differences in these longer exposures were slight and not objectionable. The loss of surface sheen or slime on the meats was only noticeable in side-by-side comparisons with the non-exposed meats. The loss of sheen gave a drier product appearance. Likewise, in these same exposures, fluid or seepage was evident about the product in the containers. The amount of seepage was minimal and expected, and it was noted in side-by-side comparisons with the controls. The slight differences noted for the

Table 25. Sensory assessment for sea scallops following exposures at various times and concentrations of acid-activated chlorine dioxide

Exposure concentration	Days in storage	Exposure time				
		0 min	1 min	10 min	60 min	180 min
Nontreated control	None	Clean, mild odor; ecru to beige color; flaccid, typical meat texture				
	1 day	No change				
	2 days	No change				
	5 days	Stronger but not objectionable odor				
Water (0 ppm)	None		NDD	NDD	NDD	NDD
	1 day		NDD	NDD	NDD	NDD
	2 days		NDD	NDD	NDD	NDD
	5 days		NDD	NDD	NDD	NDD
3.8 ppm	None		NDD	NDD	Slight loss sheen, not objectionable	Slight loss sheen, not objectionable
	1 day		NDD	NDD	No change	No change
	2 days		NDD	NDD	No change	No change
	5 days		NDD	NDD	Some odor as control	Some odor as control
11.0 ppm	None		NDD	NDD	Slight loss sheen, not objectionable	Slight loss sheen, not objectionable
	1 day		NDD	NDD	No change	No change
	2 days		NDD	NDD	No change	No change
	5 days		NDD	NDD	Some odor as control, some seepage	Some odor as control, some seepage
15.9 ppm	None		NDD	NDD	Slight loss sheen, not objectionable	Slight loss sheen, not objectionable
	1 day		NDD	NDD	No change	No change
	2 days		NDD	NDD	No change	No change
	5 days		NDD	NDD	Some odor as control, some seepage	Some odor as control, some seepage

KEY: NDD - No discernible difference from controls
No change - means no change compared to the previous day sample

longer exposure times in activated ClO_2 were not objectionable, and, in fact, improved product appearance, yet this difference was only evident in comparison with non-exposed controls.

All samples of mahi-mahi were affected by the exposure to water or test solutions (Table 26). Obviously, the exposures to water and test solutions caused color changes in the red muscle tissue from the preferred ruby-red color to a darker reddish brown. This color continued to darken for all exposed fillets. The noted bleaching effect of white tissue in samples exposed to 7.6-25.7 ppm activated ClO_2 was only discernible in side-by-side comparisons with the non-exposed controls. The degree of bleaching was not judged objectionable. The immediate red muscle color change was, however, objectionable and would reduce the value of the fillets.

Although the activated ClO_2 concentrations used for shrimp treatment were higher than those for scallops, the treatment resulted in no discernible differences for all exposure times for the first 2 days of storage (Table 27). Compared to the controls which showed obvious spoilage after 5 days of refrigeration, shrimp exposed to activated ClO_2 was less objectionable after the same storage conditions and time. The least objectionable shrimp after 5 days refrigeration was the product previously exposed to 34.9 ppm activated ClO_2 for over 60 min, even though the initial spoilage odors were obvious. Overall, exposure of shell-on shrimp in activated ClO_2 did not influence the sensory attributes of the product immediately or during subsequent refrigerated storage.

Table 26. Sensory assessment for mahi-mahi following exposures at various times and concentrations of acid-activated chlorine dioxide

Exposure concentration	Days in storage	Exposure time				
		0 min	1 min	10 min	60 min	180 min
Nontreated control	None	Clean mild fish odor; meat color off-white to beige with clean red muscle on lateral line; meat texture firm				
	1 day	Slight darkening of lateral red muscle tissue				
	2 days	Slight darkening of lateral red muscle tissue				
	5 days	More darkening of lateral red muscle tissue				
Water (0 ppm)	None		Fading in red muscle color slight objection	Fading in red muscle color slight objection	Fading in red muscle color slight objection	Fading in red muscle color slight objection
	1 day		More fading	More fading	More fading	More fading
	2 days		More fading	More fading	More fading	More fading
7.6 ppm	None		Fading as above & some bleaching of white tissue	Fading as above & some bleaching of white tissue	Fading as above & some bleaching of white tissue	Fading as above & some bleaching of white tissue
	1 day		More fading	More fading	More fading	More fading
	2 days		More fading	More fading	More fading	More fading
16.5 ppm	None		Fading & bleaching as for 0 ppm	Fading & bleaching as for 0 ppm	Fading & bleaching as for 0 ppm	Fading & bleaching as for 0 ppm
	1 day		More fading	More fading	More fading	More fading
	2 days		More fading	More fading	More fading	More fading
25.7 ppm	None		Fading & more bleaching as for 10-20 ppm	Fading & more bleaching as for 10-20 ppm	Fading & more bleaching as for 10-20 ppm	Fading & more bleaching as for 10-20 ppm
	1 day		More fading	More fading	More fading	More fading
	2 days		More fading	More fading	More fading	More fading

Table 27. Sensory assessment for penaeid shrimp following exposures at various times and concentrations of acid-activated chlorine dioxide

Exposure concentration	Days in storage	Exposure times				
		0 min	1 min	10 min	60 min	180 min
Nontreated control	None	Clean mild odor; typical shrimp meat color; meat texture firm				
	1 day	No change				
	2 days	No change				
	5 days	Strong odor objectionable				
Water (0 ppm)	None		NDD	NDD	NDD	NDD
	1 day		NDD	NDD	NDD	NDD
	2 days		NDD	NDD	NDD	NDD
	5 days		Strong odor objectionable	Strong odor objectionable	Strong odor objectionable	Strong odor objectionable
12.5 ppm	None		NDD	NDD	NDD	NDD
	1 day		NDD	NDD	NDD	NDD
	2 days		NDD	NDD	NDD	NDD
	5 days		Odor objectionable	Odor objectionable	Odor objectionable	Odor objectionable
23.3 ppm	None		NDD	NDD	NDD	NDD
	1 day		NDD	NDD	NDD	NDD
	2 days		NDD	NDD	NDD	NDD
	5 days		Odor objectionable	Odor objectionable	Odor objectionable	Odor objectionable
34.9 ppm	None		NDD	NDD	NDD	NDD
	1 day		NDD	NDD	NDD	NDD
	2 days		NDD	NDD	NDD	NDD
	5 days		Odor objectionable	Odor objectionable	Slight odor objectionable	Slight odor objectionable

KEY: NDD - No discernible difference from controls
No change - means no change compared to the previous day sample

Conclusions

Thus, ClO_2 used at more practical dose levels of ≤ 30 ppm in tap water for seafood treatment showed time- and dose-related bactericidal activity in treated solutions. The treated seafood products, in most cases, showed no discernible differences in sensory attributes to tap water treated samples. However, the ClO_2-treated shrimp showed better sensory quality than the control group after 5 days of refrigerated storage. Although low concentrations of chlorate were detected in ClO_2-treated sea scallop, mahi-mahi, and shrimp, this is not expected to cause a health concern to consumers since it will be converted to chloride during cooking. No chlorite residues were found in any of the treated seafoods.

CHAPTER VII
MUTAGENICITY EVALUATION OF THE REACTION PRODUCTS OF AQUEOUS CHLORINE DIOXIDE WITH RED GROUPER AND SALMON

Introduction

The use of chlorine dioxide (ClO_2) as a disinfectant in water treatment and the food industry has drawn great attention because of the reduced reactivity of producing possible carcinogenic/mutagenic chlorinated organic compounds from ClO_2 with organic compounds. The formation of halogenated compounds, such as trihalomethanes (THMs), during the chlorination of water and waste water containing organic substances produces new risks (Rook, 1974; Marx, 1974). In addition to chloroform, other volatile and nonvolatile mutagens of unknown identity have been produced during water chlorination. Chemical analyses of chlorinated water samples have since detected hundreds of nonvolatile chlorinated hydrocarbons of higher molecular weight, including chlorinated ketones, aldehydes, carboxylic acids, and alcohols (Stevens et al., 1990). There is increasing evidence that the major part of the toxicity of the mixture resides in the nonvolatile fraction. This diluted chemical soup is generated by the interaction of chlorine with organic matter (mostly naturally occurring humic and fulvic acids) in the untreated water. Cheh et al. (1980) found chlorinated water has an average of 5 times more mutagenic activity than untreated water. According to the findings of Kool et al. (1985), chlorine treatment of drinking water

increased the mutagenic activity in *Salmonella typhimurium* strains TA 98 and TA 100. With the exception of trihalomethanes and chlorinated acetic acids, most of the compounds occur at trace levels well below 1 ppb (Dolara et al., 1981). The presence of these compounds in treated drinking water has increased concern over the health risks to humans. In 1979, the U.S. Environmental Protection Agency (EPA) developed regulations to limit total trihalomethane levels in chlorinated drinking water to 100 μg/L (ppb).

Chlorine dioxide has been used as a chlorine alternative in water treatment plants in Europe and North America since the 1950s (Stevens et al., 1976). Miltner (1977) has reported that ClO_2 does not form trihalomethanes in drinking water. The primary products resulting from ClO_2 disinfection of surface waters include chlorites (ClO_2^-) and chlorates (ClO_3^-), which appear in concentrations of 50% and 30% of ClO_2 demand, respectively (Miltner, 1977). Metabolism studies revealed that ClO_2 is converted to chloride (Cl^-), ClO_2^-, and ClO_3^- in the rat (Abdel-Rahman et al., 1979). The ClO_2, ClO_2^-, and ClO_3^- (Cl compounds) in drinking water caused reduction in blood glutathione (GSH), a known protective agent against damage by oxidants, after 2 months of feeding to rats (Abdel-Rahman et al., 1984). Regardless of this information, research is still needed to assess the toxicity of ClO_2 reaction products in the practical commercial application situation.

Although ClO_2 is known not to form THMs when used in drinking water treatment, this may not be the case during seafood treatment. The principal precursor of THMs formation in drinking water is humic acid. However, seafoods contain complex organic compound such as proteins, lipids, vitamins, minerals, color, etc. Therefore, mutagenic reaction products can be produced following ClO_2 treatment of seafood products. Since such

information is lacking, this study was conducted to provide information necessary to critically review whether ClO_2 can be approved by regulatory agencies, such as EPA and FDA, for seafood processing. Therefore, the objective of this study was to examine the potential mutagenic activity of reaction products following treatment of seafood with ClO_2 at 20 and 200 ppm, using the Ames *Salmonella*/microsome assay. Seafood treated with aqueous chlorine at the same concentration was used as the control for comparison purpose.

Materials and Methods

Preparation of chlorine-demand-free water

Chlorine-demand-free water (CDF water) was prepared following the method of Ghanbari et al. (1982) by passing distilled water through two successive Barnstead deionizing units and then a glass column containing Porapak® Q (Supelco, Bellefonte, PA).

Generation of aqueous chlorine

The modified method of Ghanbari et al. (1983) was used to generate aqueous chlorine. Chlorine gas was generated by the dropwise addition of 8 mL of 3 N HCl solution to 4 g potassium permanganate ($KMNO_4$, Fisher Scientific, Fair Lawn, NJ) in a closed gas generation apparatus (Figure 20). Chlorine gas was trapped in 200 mL of ice cold chlorine demand-free water. Using this system, the gas was efficiently trapped as HOCl.

Preparation of activated chlorine dioxide stock solution

The chlorine dioxide stock solution was prepared by mixing 1 mL 85% phosphoric acid (Fisher Scientific, Certified ACS) for 5 min at room temperature with 20 mL Oxine® concentrate (Bio-Cide International, Inc., Norman, OK) in a brown flask sealed with a glass stopper. The reaction mixture was then diluted with 9 volumes of CDF water. This stock

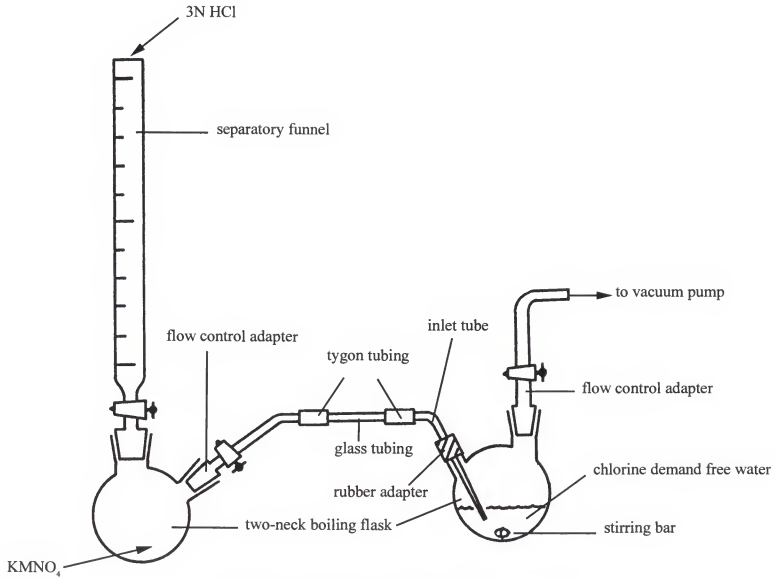


Figure 20. Apparatus for aqueous chlorine generation

solution was used to prepare test solutions (20 and 200 ppm ClO_2) with brine based on the content of total available ClO_2 (TACD expressed as μg/mL ClO_2) as determined by iodometric titration (APHA, 1989). The concentrations of total available chlorine (TAC), TACD, and various chlorine species in stock and test solutions were determined by iodometric and *N, N*-Diethyl-*p*-phenylenediamine (DPD) ferrous titration methods (APHA, 1989). The stock and test solutions used to treat red grouper and salmon were freshly prepared on the day of testing.

Sample preparation

Fresh Atlantic salmon (*Salmo salar*) and red grouper (*Epinephelus morio*) were purchased from a local seafood store. The fish was filleted and skinned, and the fillets were cut into cubes (2.5 x 2.5 x 2.5 cm). Fish cubes were mixed well and randomly sampled (60 g) for treatment with five volumes (300 mL) of aqueous Cl_2, a ClO_2 solution or brine (1:5 w/v). The treated aqueous solutions and fish cubes were collected and processed to prepare samples for mutagenicity testing.

The outer surfaces of the treated fish cubes were cut off using a knife, and 30 g of this tissue were then chopped, minced, and homogenized in a Waring blender (Dynamics Corporation of America, New Hartford, CT) with 60 mL distilled water. The homogenate was extracted with 2 volumes (180 mL) of a methylene chloride (CH_2Cl_2)/methanol (CH_3OH) mixture (1:1, v/v), and the mixture was filtered through a Whatman #1 filter paper (Figure 21). The retained solid samples were homogenized in the blender two more times with 100 mL each of CH_2Cl_2/CH_3OH mixture and filtered again. The filtrates were pooled in a separatory funnel, and the bottom (CH_2Cl_2) layer was removed and retained. After the

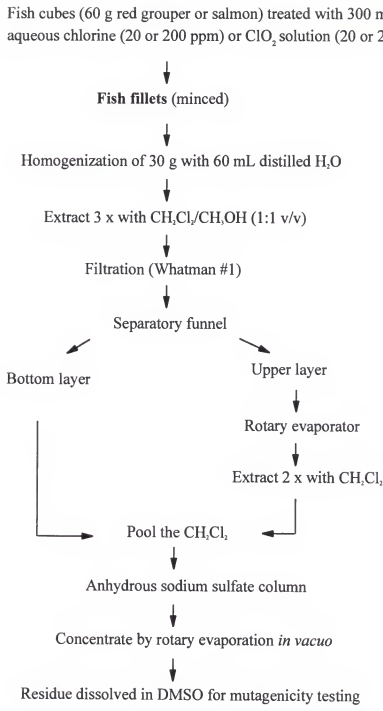


Figure 21. Schematic presentation of sample preparation from treated fish cubes for mutagenicity assay

methanol in the aqueous phase was removed using a rotary evaporator, the aqueous phase was extracted two times with equal volumes of CH_2Cl_2. All CH_2Cl_2 extracts were pooled and passed through a column of anhydrous sodium sulfate. After removing CH_2Cl_2 using a rotary evaporator *in vacuo*, the residue was dissolved in small volume of CH_2Cl_2 and the solution transferred to a pre-weighed amber threaded vial (1.8 mL) with a rubber lined closure. The CH_2Cl_2 in the vials was flushed to dryness under a stream of nitrogen gas and the weight measured again for determination of sample weight. The sample was then properly diluted with spectrophotometric-grade dimethylsulfoxide (DMSO, Schwarz/Mann Biotech, Cleveland, OH) for mutagenicity test.

The treated aqueous chlorine, chlorine dioxide solutions, and brine (300 mL each) solutions were collected after reacting with fish cubes for 5 min, and extracted with $CH_2Cl_2/CHCl_3$ mixture following the procedure shown in Figure 22. The pooled CH_2Cl_2 extracts were processed, and residues transferred to vials, then dissolved and diluted with an appropriate amount of DMSO for mutagenicity testing.

Bacterial cultures

Salmonella typhimurium TA 98 and TA 100 were provided by Dr. B.N. Ames of the Department of Biochemistry, University of California, Berkeley, CA. The tester strains were stored at -80 °C. They were prepared from the overnight cultures in Oxoid nutrient broth No. 2 (Oxoid Co., Vasingstoke, Hant, England) to a density of 10^8 bacteria per mL. For each 1 mL of culture, 0.09 mL of DMSO was added as a cryoprotective agent. The culture was distributed aseptically into sterile 1 mL cryogenic vials (Nalge Co, Rochester, NY). The vials were filled near to capacity to eliminate air space at the top which helped to minimize

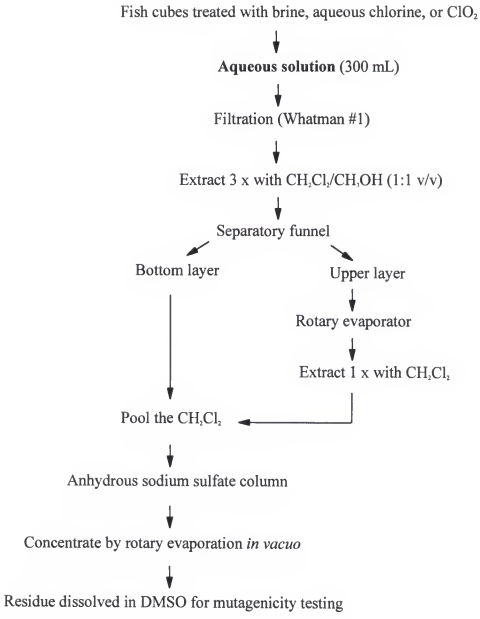


Figure 22. Schematic presentation of sample preparation of treated aqueous solution for mutagenicity assay

oxidative damage. The vials were chilled at -5 °C until the cultures were frozen and then transferred to a -80 °C freezer. The tester strains were checked routinely to confirm their genetic features using the procedure of Ames et al. (1975).

Master plates were used as the source of bacterial inocula for preparing test cultures. The master plate was prepared by placing one drop of thawed culture on the surface of a bottom agar plate (Vogel-Bonner agar medium) containing 70 µL of 0.2 M histidine, 70 µL of 1 mM biotin and 50 µL (0.8 mg) of ampicillin. The culture was streaked out for single colony isolates using a sterile platinum wire. It was incubated for 48 hrs at 37 °C and stored at 4 °C.

One day prior to performing the mutagenicity assay, isolated colonies of the test strains (TA 98 and TA 100) were picked up from master plates using a sterile platinum wire and inoculated into Oxoid nutrient broth No. 2. The cultures were grown overnight at 35 °C in a shaking water bath (Gyrotory® water bath shaker, Model G76, New Brunswick Scientific Co., Edison, NJ) at 100 rpm.

Metabolic activation system

A rat liver S-9 preparation (Aroclor 1254-induced male Sprague-Dawley liver in 0.154 M KCl solution) was purchased from Molecular Toxicology, Inc. (Boone, NC) and stored at -70 °C. Before use, the rat liver S-9 was thawed immediately and mixed with an NADPH-generating system comprising of 4 mM NADP, 5 mM glucose-6-phosphate, 8 mM $MgCl_2$, 33 mM KCl and 100 mM sodium phosphate (pH 7.4) (Maron and Ames, 1983). The S-9 mix was then filtered through a 0.8, 0.45, and 0.2-µm Nalgene disposable filter (Nalge Co., Rochester, NY) and its sterility checked in nutrient broth.

The Ames *Salmonella*/microsome assay

The standard techniques of Ames et al. (1975) and Maron and Ames (1983) were used. The assay was performed using the two most sensitive *Salmonella typhimurium* strains TA 98 and TA 100. The test was done with and without rat metabolic activation system (S-9 mix). The S-9 mix provided a broad spectrum of enzyme activities for activating the test compounds. The experiments were repeated at least twice.

Four plates were used for each sample dose. For the plate incorporation test, 0.1 mL of a fresh culture of the tester bacteria (10^8 CFU/mL) was added along with a pre-determined dose of the test sample (25 µL) into 2.5 mL of the top agar containing 0.5 mM of histidine and biotin (Sigma Chemical, St. Louis, MO) (Figure 23). For tests requiring metabolic activation, 0.5 mL of S-9 mix was also added; while in tests without metabolic activation it was replaced with 0.5 mL of 0.25 M sodium phosphate buffer (pH 7.4). After vortex mixing, the test mixture was poured onto bottom agar plates containing essential minerals, a carbon and nitrogen source for bacterial growth, which helps to express revertant colonies. Concurrent positive and negative controls were included in all assays. 2-Aminofluorene (Aldrich, Milwaukee, WI) was used for both strains in the presence of the S-9 mix. Methylmethane sulfonate (MMS, Aldrich) was used for strain TA 100 and 2-nitrofluorene (2-NF, Aldrich) for TA 98 when S-9 mix was absent. Negative controls which contained the bacteria, S-9 mix (or buffer) and DMSO were required to establish spontaneous revertant numbers for each tester strain. The plates were incubated in an inverted position at 37 °C for 2 days before counting the revertant colonies. The mutagenicity ratio, the number of revertants per test sample divided by revertants per controls, was used as indication of

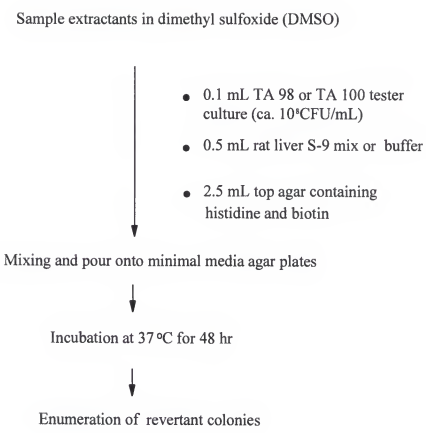


Figure 23. Protocol for Ames *Salmonella*/microsome assay

mutagenic potency. A ratio of 2 or more is generally considered positive in the assay (Ames et al., 1975).

Results and Discussion

Table 28 shows the amounts of organic solvent extractable reaction products from treated red grouper and salmon, and the reaction solutions. The higher fat content of salmon contributed to the greater weight of fish extracts than those of red grouper. The characteristic reversion of tester strains to some diagnostic mutagens (positive controls) and negative controls are shown in Table 29.

The results of the mutagenicity testing are shown in Tables 30- 33. The organic solvent extracts of brine-treated samples (both the fish and the solution) showed no mutagenic activity towards *S. typhimurium* TA 98 or TA 100 with or without rat S-9 mix. Only the extracts from the treated 200 ppm aqueous chlorine solution with red grouper and salmon showed weak mutagenic activity toward *S. typhimurium* TA 100 (Table 31). The addition of S-9 mix did not enhance the mutagenic activity. Such extracts, however, failed to induce mutation in strain TA 98. Therefore, the reaction products are capable of causing base-pair substitution mutations. The mutagenicity of the reaction products to TA 100 was decreased in the presence of the S-9 mix. The binding of the test sample with the S-9 enzyme system may have decreased the availability of the test sample for interaction with bacterial DNA and induction of mutation. The reaction products obtained from the interaction of ClO_2 either at 20 or 200 ppm with red grouper and salmon were not mutagenic to either tester strain in the presence or absence of the S-9 mix (Tables 32 and 33). The

Table 28. Net weights of organic solvent extracts of the treated seafood and solutions following interaction with various chlorine and chlorine dioxide solutions

Treatment	Red grouper		Salmon	
	solution extract	fish extract	solution extract	fish extract
brine (control)	0.01 g	0.72 g	0.02 g	3.65 g
20 ppm aqueous chlorine	0.02 g	0.99 g	0.01 g	6.69 g
200 ppm aqueous chlorine	0.02 g	1.14 g	0.13 g	4.67 g
20 ppm ClO_2 solution	0.04 g	0.54 g	0.04 g	5.14 g
200 ppm ClO_2 solution	0.02 g	1.18 g	0.05 g	3.11 g

Sixty grams of fish cubes were treated with 300 mL of chlorinating solutions for 5 min. After the outer layers of the treated fish cubes were cut off and minced, 30 g were extracted with CH_2Cl_2/CH_3OH mixture to prepare the test samples. The treated solutions (300 mL) were also extracted similarly to prepare the other set of test samples.

Table 29. Reversion properties of *Salmonella typhimurium* TA 98 and TA 100 to negative controls and positive mutagen controls

Sample	Dose/plate	TA 98		TA 100	
		-S9	+S9	-S9	+S9
Bacteria + Buffer	25 µL	19 ± 5	22 ± 2	84 ± 6	102 ± 13
Bacteria + DMSO	25 µL	21 ± 4	23 ± 7	80 ± 7	90 ± 13
Bacteria + Mutagen					
MMS (5 µL/25 µL)	30 µL	--	--	2130 ± 61	--
2-NF (5 µg/25 µL)	30 µL	2544 ± 154	--	--	--
2-AF (10 µg/25 µL)	30 µL	--	9968 ± 408	--	4731 ± 321

MMS, 2-AF, and 2-NF were dissolved in DMSO (dimethylsulfoxide).
MMS = methylmethanesulfonate, 2-AF = 2-aminofluorene, and 2-NF = 2-nitrofluorene.

Table 30. Mutagenicity testing with *Salmonella typhimurium* strain TA 98 of the organic solvent extracts from treated aqueous chlorine and chlorine dioxide solutions following interaction with red grouper or salmon cubes

Sample	Test dose[a] (per plate)	Number of revertants/plate[b]			
		TA 98			
		-S9	MR[c]	+S9	MR
Red grouper					
Brine	250 µg	18 ± 4	1	19 ± 4	1
20 ppm Cl_2	500 µg	21 ± 5	1.17	20 ± 7	1.05
200 ppm Cl_2	500 µg	27 ± 8	1.5	26 ± 4	1.39
20 ppm ClO_2	500 µg	18 ± 2	1	25 ± 4	1.32
	250 µg	20 ± 7	1.11	24 ± 6	1.26
200 ppm ClO_2	500 µg	18 ± 6	1	24 ± 4	1.26
Salmon					
Brine	500 µg	16 ± 4	1	21 ± 3	1
20 ppm Cl_2	250 µg	16 ± 3	1	23 ± 4	1.10
200 ppm Cl_2	2 mg	10 ± 3	0.63	24 ± 6	1.14
	1 mg	27 ± 7	1.69	29 ± 6	1.38
	0.5 mg	26 ± 4	1.63	20 ± 6	0.95
20 ppm ClO_2	500 µg	17 ± 5	1.06	23 ± 5	1.10
	250 µg	17 ± 4	1.06	21 ± 3	1
200 ppm ClO_2	500 µg	20 ± 4	1.25	22 ± 7	1.05
	250 µg	20 ± 10	1.25	21 ± 5	1

[a]The test dose per plate is the amount of organic solvent extract from the treated reaction solution

[b]Mean ± standard deviation from eight plates of duplicate trials.

[c]Mutagenicity ratio (MR) is the number of revertants per test dose divided by revertants per controls.

Table 31. Mutagenicity testing with *Salmonella typhimurium* strain TA 100 of the organic solvent extracts from treated aqueous chlorine and chlorine dioxide solutions following interaction with red grouper or salmon cubes

Sample	Test dose[a] (per plate)	Number of revertants/plate[b]			
		TA 100			
		-S9	MR[c]	+S9	MR
Red grouper					
Brine	250 µg	80 ± 10	1	97 ± 13	1
20 ppm Cl_2	500 µg	84 ± 16	1.05	92 ± 10	0.95
200 ppm Cl_2	500 µg	342 ± 79	4.28	238 ± 59	2.45
20 ppm ClO_2	500 µg	83 ± 11	1.04	97 ± 8	1
	250 µg	80 ± 9	1	106 ± 20	1.09
200 ppm ClO_2	500 µg	82 ± 7	1.03	101 ± 10	1.04
Salmon					
Brine	500 µg	79 ± 5	1	107 ± 11	1
20 ppm Cl_2	250 µg	78 ± 9	0.99	105 ± 22	0.98
200 ppm Cl_2	2 mg	238 ± 21	3.01	263 ± 24	2.46
	1 mg	161 ± 35	2.04	179 ± 23	1.67
	0.5 mg	146 ± 40	1.85	137 ± 14	1.28
20 ppm ClO_2	500 µg	89 ± 8	1.13	95 ± 11	0.89
	250 µg	84 ± 3	1.06	96 ± 14	0.90
200 ppm ClO_2	500 µg	171 ± 24	2.16	114 ± 20	1.07
	250 µg	112 ± 19	1.42	94 ± 12	0.88

[a]The test dose per plate is the amount of organic solvent extract from the treated reaction solution

[b]Mean ± standard deviation from eight plates of duplicate trials.

[c]Mutagenicity ratio (MR) is the number of revertants per test dose divided by revertants per controls.

Table 32. Mutagenicity testing with *Salmonella typhimurium* strain TA 98 and TA 100 of the organic solvent extracts from treated red grouper with aqueous chlorine and chlorine dioxide

Sample	Test dose[a] (per plate)	Number of revertants/plate[b]			
		TA 98		TA 100	
		-S9	+S9	-S9	+S9
Red grouper					
Brine	5 mg	28 ± 3	26 ± 5	107 ± 19	112 ± 16
	1 mg	24 ± 3	26 ± 5	121 ± 19	108 ± 17
	200 µg	27 ± 6	27 ± 3	106 ± 21	122 ± 23
	40 µg	26 ± 3	28 ± 6	111 ± 14	109 ± 23
20 ppm Cl_2	5 mg	33 ± 4	34 ± 6	113 ± 20	103 ± 10
	1 mg	25 ± 3	24 ± 6	117 ± 18	107 ± 13
	200 µg	24 ± 3	25 ± 4	114 ± 20	113 ± 26
	40 µg	24 ± 4	23 ± 3	103 ± 20	120 ± 22
200 ppm Cl_2	5 mg	40 ± 10	37 ± 7	115 ± 35	118 ± 11
	1 mg	29 ± 6	30 ± 6	116 ± 30	123 ± 9
	200 µg	30 ± 12	27 ± 4	116 ± 21	122 ± 16
	40 µg	26 ± 5	25 ± 6	116 ± 19	117 ± 27
20 ppm ClO_2	5 mg	30 ± 7	30 ± 3	117 ± 24	111 ± 20
	1 mg	26 ± 6	29 ± 4	114 ± 20	118 ± 16
	200 µg	25 ± 5	24 ± 5	113 ± 24	106 ± 21
	40 µg	25 ± 5	23 ± 7	105 ± 24	116 ± 26
200 ppm ClO_2	5 mg	25 ± 5	30 ± 4	115 ± 29	117 ± 20
	1 mg	23 ± 5	29 ± 8	112 ± 30	117 ± 22
	200 µg	28 ± 8	24 ± 5	106 ± 15	119 ± 24
	40 µg	25 ± 2	24 ± 3	108 ± 16	122 ± 15

[a]The test dose per plate is the amount of organic solvent extract of the treated fish.
[b]Mean ± standard deviation from eight plates of duplicate trials.

Table 33. Mutagenicity testing with *Salmonella typhimurium* strain TA 98 and TA 100 of the organic solvent extracts from treated salmon with aqueous chlorine and chlorine dioxide

Sample	Test dose[a] (per plate)	Number of revertants/plate[b]			
		TA 98		TA 100	
		-S9	+S9	-S9	+S9
Salmon					
Brine	5 mg	23 ± 5	26 ± 7	103 ± 17	117 ± 18
	1 mg	21 ± 5	25 ± 6	111 ± 23	116 ± 16
	200 µg	21 ± 2	26 ± 4	115 ± 32	114 ± 27
	40 µg	20 ± 4	26 ± 6	106 ± 30	118 ± 25
20 ppm Cl_2	5 mg	22 ± 5	28 ± 5	109 ± 27	122 ± 20
	1 mg	21 ± 3	25 ± 5	107 ± 20	126 ± 19
	200 µg	21 ± 5	26 ± 5	109 ± 25	120 ± 26
	40 µg	20 ± 5	24 ± 3	108 ± 30	118 ± 24
200 ppm Cl_2	5 mg	24 ± 7	24 ± 6	105 ± 20	109 ± 20
	1 mg	23 ± 6	22 ± 4	100 ± 13	109 ± 14
	200 µg	24 ± 4	27 ± 9	104 ± 19	117 ± 16
	40 µg	22 ± 5	30 ± 8	112 ± 24	117 ± 24
20 ppm ClO_2	5 mg	26 ± 6	29 ± 7	104 ± 22	110 ± 16
	1 mg	21 ± 4	28 ± 4	107 ± 28	122 ± 26
	200 µg	23 ± 7	30 ± 7	110 ± 22	112 ± 19
	40 µg	20 ± 4	27 ± 6	98 ± 21	112 ± 12
200 ppm ClO_2	5 mg	24 ± 4	27 ± 5	105 ± 20	108 ± 15
	1 mg	21 ± 3	24 ± 5	104 ± 27	120 ± 20
	200 µg	22 ± 4	26 ± 6	104 ± 24	116 ± 21
	40 µg	23 ± 4	27 ± 6	100 ± 22	104 ± 15

[a]The test dose per plate is the amount of organic solvent extract of the treated fish.
[b]Mean ± standard deviation from eight plates of duplicate trials.

extracts of the treated ClO_2 solutions also showed no mutagenic activity towards either tester strain (Tables 30 and 31).

Conclusions

Thus, unlike aqueous chlorine, no mutagenic reaction products were produced following treatment of red grouper and salmon with aqueous ClO_2 at 20 or 200 ppm. Since ClO_2 is a potent bactericidal agent, is shown to improve the quality of seafood products, and is apparently safe, it can be used as a processing aid in the seafood industry.

CHAPTER VIII
SUMMARY AND RECOMMENDATIONS

Due to the perishable nature of seafood, the seafood industry has long applied aqueous chlorine solution for washing fish, shrimp, crab, clam, and oyster to meet microbiological standards, prevent spoilage, and increase shelf-life. However, the bactericidal ability of a chlorine solution decreases at high pH and in the presence of high levels of organic matter. Moreover, potential mutagenic/carcinogenic reaction products, such as trihalomethanes (THMs), are produced following chlorine treatment. These potentially toxic compounds may pose health hazards to consumers. Such safety concerns over the production of harmful organic by-products during chlorination have prompted the research for chlorine alternatives. Chlorine dioxide (ClO_2), a potent antimicrobial agent, is one such alternative.

In the study to evaluate the effectiveness of ClO_2 treatment on quality of seafood products, it was found that treatment of red grouper and salmon fillets, scallops, shrimps, and whole salmon and grouper with ClO_2 solutions on day 0 caused dose-related decreases in numbers of natural flora. The treated groups with ClO_2 followed by cold storage (4 °C) for 3 and 7 days also had less bacterial numbers than the nontreated and brine treated groups. The treated ClO_2 solutions also contained very low or no bacterial loads. The fish fillets treated with 20 or 40 ppm ClO_2 were usually of very good quality (Grade A), showing no discoloration or defects in appearance. Treatment with ClO_2 is thus very helpful in resolving

odor problems of seafood. Discoloration, however, occurred with seafood treated with 100 or 200 ppm ClO_2. The skin of the treated red grouper and salmon became lighter in color, and a chocolate color occurred on the gills due to oxidation of blood by ClO_2.

Treatment of salmon and red grouper with ClO_2 caused slight elevation of thiobarbituric acid (TBA) values. Such treatment did not cause any obvious difference in fish fatty acid compositions and protein contents, although differences occurred with some individual fatty acids. Treatments with ClO_2 also caused a reduction of thiamine content in salmon and red grouper, and riboflavin in red grouper. The calcium, iron and phosphorus content of red grouper fillets, and phosphorus and potassium content of salmon fillets were not affected by treatment with ClO_2 solution.

The lower reactivity of ClO_2 compared to HOCl is partially due to its lower redox potential, 0.95 V for ClO_2 compared to 1.49 V for HOCl (Masschelein, 1979). Although ClO_2 has a greater oxidizing potential than HOCl, the oxidizing power of ClO_2 is not readily available under the experimental conditions owing to its reduction to ClO_2^-, which has an even lower redox potential (0.78 V). The ClO_2^- produced comprises 80% of the oxidizing capacity of ClO_2. Therefore, the lower reactivity of ClO_2 with organic molecules could produce less oxidation products in treated seafood. As a result of its lower reactivity, it may have an increased bactericidal effectiveness in treating or stabilizing water in food processing operations or seafoods.

Although THMs are generally not formed by the action of ClO_2, other oxidized reaction products and potentially mutagenic compounds can be formed following ClO_2 treatment of seafood organic matter. Furthermore, as ClO_2 disproportionates in water, both

chlorite (ClO_2^-) and chlorate (ClO_3^-) are formed as by-products. Only low levels of chlorate residues were detected in some of the treated sea scallops, mahi-mahi, and shrimp. No chlorite residues were found in any of the treated seafoods. The mutagenicity testing with Ames *Salmonella*/microsome assay showed that the reaction products from both treated ClO_2 solution and fish were not mutagenic to either tester strain. Finally, since ClO_2 is a more potent bactericidal agent and is less reactive with organic matter, and since the reaction products are non-mutagenic, toward *S. typhimurium* tester strains TA 98 and TA 100, it could be used as a chlorine alternative to maintain quality/safety of seafood products. Such treatment with ClO_2 is expected to enhance the freshness, extend the shelf-life, and improve the safety of seafood. It is important that the results of these studies be reviewed by government regulatory agencies to assist in their evaluation for approval of ClO_2 usage in the seafood industry.

The ClO_2 solution could potentially find extensive application in the seafood industry. For example, seafood products can be submerged in a water tank containing icy ClO_2 water maintained at a constant concentration (preferably < 40 ppm) to preserve the freshness and lengthen the shelf-life. Even though large quantities of seafood could share this washing tank, the constant ClO_2 concentration can kill the bacterial load in the water and prevent the spread of bacterial contamination from a particular piece of seafood to others. Fish can be washed in ClO_2 solution before scaling, and shrimp can be dipped in ClO_2 solution prior to freezing for maintaining a better quality. In addition, ClO_2 can be used as a sanitizing agent for cleaning knives and utensils and for workers to wash their hands. Chlorine dioxide can also be applied in the depuration system to reduce seafood pathogens.

APPENDIX

Table A-1. Sensory evaluation sheet for determination of the quality of red grouper or salmon fillets

Fish fillet: Date: No.

Defect	Degree	A1	B1	C1	D1	E1	F1
Appearance defects (flesh consistency)	Slight						
	Moderate						
	Excessive						
Discoloration	Slight						
	Moderate						
	Excessive						
Odor	Slight						
	Moderate						
	Excessive						
Defect	Degree	A2	B2	C2	D2	E2	F2
Appearance defects (flesh consistency)	Slight						
	Moderate						
	Excessive						
Discoloration	Slight						
	Moderate						
	Excessive						
Odor	Slight						
	Moderate						
	Excessive						
Defect	Degree	A3	B3	C3	D3	E3	F3
Appearance defects (flesh consistency)	Slight						
	Moderate						
	Excessive						
Discoloration	Slight						
	Moderate						
	Excessive						
Odor	Slight						
	Moderate						
	Excessive						

Table A-2. Sensory evaluation sheet for determination of the quality of scallops or shrimp

Sample: Date: No.

Defect	Degree	A1	B1	C1	D1	E1	F1
Discoloration	Slight						
	Moderate						
	Excessive						
Odor	Slight						
	Moderate						
	Excessive						
Defect	Degree	A2	B2	C2	D2	E2	F2
Discoloration	Slight						
	Moderate						
	Excessive						
Odor	Slight						
	Moderate						
	Excessive						
Defect	Degree	A3	B3	C3	D3	E3	F3
Discoloration	Slight						
	Moderate						
	Excessive						
Odor	Slight						
	Moderate						
	Excessive						

Table A-3. Sensory evaluation sheet for determination of the quality of red grouper or salmon (whole fish)

Fish: Date: No.

Defect	Degree	A1	B1	C1	D1	E1	F1	A2	B2	C2	D2	E2	F2	A3	B3	C3	D3	E3	F1
Appearance defects (flesh consistency)	Slight																		
	Moderate																		
	Excessive																		
Eyes	Slight																		
	Moderate																		
	Excessive																		
Body damage	Slight																		
	Moderate																		
	Excessive																		
Gills and gut cavity	Slight																		
	Moderate																		
	Excessive																		
Odor	Slight																		
	Moderate																		
	Excessive																		
Skin discoloration	Slight																		
	Moderate																		
	Excessive																		
Belly flaps	Slight																		
	Moderate																		
	Excessive																		

Table A-4. Score sheet for grading red grouper or salmon fillet

Sample:		Treatment:										Experiment No.										Day:										Date:									
Fish No.		I										II										III																	
Panelist No.		1	2	3	4	5	6	7	8	9	10	1	2	3	4	5	6	7	8	9	10	1	2	3	4	5	6	7	8	9	10										
Appearance defects (flesh consistency)	Slight																																							
	Moderate																																							
	Excessive																																							
Discoloration	Slight																																							
	Moderate																																							
	Excessive																																							
Odor	Slight																																							
	Moderate																																							
	Excessive																																							
Total serious																																								
Subsample grade																																								
Total grade A																																								
Total grade B																																								
Total substandard																																								
Total sample grade																																								
Remarks																																								

Table A-5. Score sheet for grading scallops or shrimp

Sample:		Treatment:										Experiment No.										Day:										Date:									
Fish No.		I										II										III																	
Panelist No.		1	2	3	4	5	6	7	8	9	10	1	2	3	4	5	6	7	8	9	10	1	2	3	4	5	6	7	8	9	10										
Discoloration	Slight																																								
	Moderate																																								
	Excessive																																								
Odor	Slight																																								
	Moderate																																								
	Excessive																																								
Total minor																																								
Total major																																								
Total serious																																								
Subsample grade																																								
Total grade A																																								
Total grade B																																								
Total substandard																																								
Total sample grade																																								
Remarks																																								

Table A-6. Score sheet for grading red grouper or salmon

Sample:		Treatment:										Experiment No.					Day:					Date:									
Fish No.		I										II										III									
Panelist No.		1	2	3	4	5	6	7	8	9	10	1	2	3	4	5	6	7	8	9	10	1	2	3	4	5	6	7	8	9	10
Appearance defects (Flesh consistency)	slight																														
	moderate																														
	excessive																														
Eyes	slight																														
	moderate																														
	excessive																														
Body damage	slight																														
	moderate																														
	excessive																														
Gills and gut cavity	slight																														
	moderate																														
	excessive																														
Odor	slight																														
	moderate																														
	excessive																														
Skin discoloration	slight																														
	moderate																														
	excessive																														
Belly flaps	slight																														
	moderate																														
	excessive																														
Total minor																															
Total major																															
Total serious																															
Subsample grade																															
Total grade A																															
Total grade B																															
Total substandard																															
Total sample grade																															
Remarks																															

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